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L3: Entry 1 of 3 APP# 08/894,626 File: USPT Mar 12, 2002

WO 96/25941

DOCUMENT-IDENTIFIER: US 6355780 B1
TITLE: Antibodies to the death domain motifs of regulatory proteins

Abstract Text (1):
A modulator of regulatory cellular events occurring intracellularly which are mediated by regulatory proteins containing a "death domain" motif is provided. The "death domain" is a regulatory portion of the regulatory proteins, and the modulator is capable of interacting with one or more "death domain" motifs contained in the regulatory proteins and affecting the regulatory action of one or more of the regulatory proteins. The modulator preferably is capable of interating with "death domain" motifs within p55-TNF-R, FAS/APO1-R, NGF-R, MORT-1, RIP, TRADD, or ankryin, as illustrated in the Figure. A method for producing the modulators is also provided. The modulators are useful for modulating functions mediated in cells by proteins containing the "death domain".

Brief Summary Text (2):
The present invention is generally in the field of regulatory proteins which exert their effects by intracellular signaling processes which are mediated by regulatory elements (domains or motifs) contained within the intracellular domains of these proteins. More specifically, the present invention concerns new modulators being proteins, peptides, antibodies or analogs or fragments of any thereof, and organic compounds which are capable of interacting with, or binding to the newly discovered `death domain` motif present in a wide range of related and unrelated proteins, for example, receptors of the TNF/NGF family such as p55 TNF-R, FAS-R, NGF-R, a related protein MORT-1, proteins known as TRADD and RIP and the unrelated protein ankyrin 1. These new modulators are capable of modulating or regulating the activity of the proteins which contain the `death domain` motif.

Brief Summary Text (4):
There is a very large group of regulatory proteins which exert their regulatory effects on cells by way of intracellular signaling processes, mediated by regulatory portions or motifs contained within these proteins. Members of this group of proteins include, receptors belonging to the TLF/NGF family of receptors, such as, for example, the p55 and p75 TNF receptors (p55 and p75 TNF-Rs), the NGF receptor (NGF-R) and the Fas/APO1 protein (also called the FAS-ligand receptor or FAS-R, and hereinafter will be called FAS-R); these receptors being characterized by having an extracellular ligand-binding domain, a transmembrane domain and an intracellular (IC) domain, which intracellular domain, or portions thereof, is involved in the mediation of the intracellular signaling events initiated by the binding of the ligand to the extracellular domain. Other members of this group include various intracellular proteins, for example, the cytoskeleton-associated structural proteins, the ankyrins, which have a regulatory domain that is possibly involved in the ability of these proteins to associate with or bind to other cytoskeletal proteins, e.g. spectrin, or to other transmembrane proteins. Yet another member of this group is the recently identified MORT1 protein (also called HF1, see co-pending IL 112002 and EL 112692), which is capable of binding specifically to the intracellular domain of the FAS-R, and which is also capable of self-association and of mediating, in a ligand-independent manner, cytotoxic effects on cells. In MORT-1, a regulatory domain was also identified (see IL 112692).

Brief Summary Text (9):
Another member of the TNF/NGF superfamily of receptors is the FAS receptor (FAS-R) which has also been called the Fas antigen, a cell-surface protein expressed in various tissues and sharing homology with a number of cell-surface receptors including TNF-R and NGF-R. The FAS-R mediates cell death in the form of apoptosis (Itoh et al., 1991), and appears to serve as a negative selector of autoreactive T cells, i.e. during maturation of T cells, FAS-R mediates the apoptopic death of T cells recognizing self-antigens. It has also been found that mutations in the FAS-R gene (lpr) cause a lymphoproliferation disorder in mice that resembles the human autoimmune disease systemic lupus erythematosus (SLE) (Watanabe-Fukunaga et al., 1992). The ligand for the FAS-R appears to be a cell-surface associated molecule carried by, amongst others, killer T cells (or cytotoxic T lymphocytes--CTLs), and hence when such CTLs contact cells carrying FAS-R, they are capable of inducing apoptopic cell death of the FAS-R-carrying cells. Further, a monoclonal antibody has been prepared that is specific for FAS-R, this monoclonal antibody being capable of inducing apoptopic cell death in cells carrying FAS-R, including mouse cells transformed by cDNA encoding human FAS-R (Itoh et al., 1991).

Brief Summary Text (10):
It has also been found that various other normal cells, besides T lymphocytes, express the FAS-R on their surface and can be killed by the triggering of this receptor. Uncontrolled induction of such a killing process is suspected to contribute to tissue damage in certain diseases, for example, the destruction of liver cells in acute hepatitis. Accordingly, finding ways to restrain the cytotoxic activity of FAS-R may have therapeutic potential.

Brief Summary Text (11):
Conversely, since it has also been found that certain malignant cells and HIV-infected cells carry the FAS-R on their surface, antibodies against FAS-R, or the FAS-R ligand, may be used to trigger the FAS-R mediated cytotoxic effects in these and thereby provide a means for combating such malignant cells or HFV-infected cells (see Itoh et al., 1991). Finding yet other ways for enhancing the cytotoxic activity of FAS-R may therefore also have therapeutic potential.

Brief Summary Text (12):
In co-pending IL 109632, IL 111125 and IL 112002 there is described that the intracellular domain of FAS-R, the so-called FAS-IC, is capable of self-association and contains within this intracellular domain a region called the `death domain` (DD) which is primarily responsible for the self-association of the FAS-IC. This `death domain` shares sequence homology with the p55 TNF-R, `death domain` (p55DD).

Brief Summary Text (13):
It has been a long felt need to provide a way for modulating the cellular response to TNF (.alpha. or .beta.) and FAS-R ligand, for example, in pathological situations as mentioned above, where TNF or FAS-R ligand is over-expressed it is desirable to inhibit the TNF- or FAS-R ligand-induced cytocidal effects, while in other situations, e,g. wound healing applications, it is desirable to enhance the TNF effect, or in the case of FAS-F, in tumor cells or HIV-infected cells it is desirable to enhance the FAS-R mediated effect.

Brief Summary Text (14):
A number of approaches have been made by the present inventors (see for example, European Application Nos. EP 186833, EP 308378, EP 398327 and EP 412486) to regulate the deleterious effects of TNF by inhibiting the binding of TNF to its receptors using anti-TNF antibodies or by using soluble TNF receptors (being essentially the soluble extracellular domains of the receptors) to compete with the binding of TNF to the cell surface-bound TNF-Rs. Further, on the basis that TNF-binding to its receptors is required for the TNF-induced cellular effects, approaches by the present inventors (see for example IL 101769 and its corresponding EP 568925) have been made to modulate the TNF effect by modulating the activity of the TNF-Rs. Briefly, EP 568925 (IL 101769) relates to a method of modulating signal transduction and/or cleavage in TNF-Rs whereby peptides or other molecules may interact either with the receptor itself or with effector proteins interacting with the receptor, thus modulating the normal functioning of the TNF-Rs. In EP 568925 there is described the construction and characterization of various mutant p55 TNF-Rs, having mutations in the extracellular, transmembranal, and intracellular domains of the p55 TNF-R. In this way regions within the above domains of the p55 TNF-R were identified as being essential to the functioning of the receptor, i.e. the binding of the lizand (TNF) and the subsequent signal transduction and intracellular signaling which ultimately results in the observed TNF-effect on the cells. Further, there is also described a number of approaches to isolate and identify proteins, peptides or other factors which are capable of binding to the various regions in the above domains of the TNF-R, which proteins, peptides and other factors may be involved in regulating or modulating the activity of the TNF-R. A number of approaches for isolating and cloning the DNA sequences encoding such proteins and peptides; for constructing expression vectors for the production of these proteins and peptides; and for the preparation of antibodies or fragments thereof which interact with the TNF-R or with the above proteins and peptides that bind various regions of the TNF-R, are also set forth in EP 568925. However, no description is made in EP 568925 of the actual proteins and peptides which bind to the intracellular domains of the TNF-Rs (e.g. p55 TNF-R), nor is any description made of the yeast two-hybrid approach to isolate and identify such proteins or peptides which bind to the intracellular domains of TNF-Rs. Similarly, heretofore there has been no disclosure of proteins or peptides capable of binding the intracellular domain of FAS-R.

Brief Summary Text (15):
Thus, when it is desired to inhibit the effect of TNF, or the FAS-R ligand, it would be desirable to decrease the amount or the activity of TNF-Rs or FAS-R at the cell surface, while an increase in the amount or the activity of TNF-Rs or FAS-R would be desired when an enhanced TNF or FAS-R ligand effect is sought. To this end the promoters of both the p55 TNF-R and the p75 TNF-R have been sequenced, analyzed and a number of key sequence motifs have been found that are specific to various transcription regulating factors, and as such the expression of these TNF-Rs can be controlled at their promoter level, i.e. inhibition of transcription from the promoters for a decrease in the number of receptors, and an enhancement of transcription from the promoters for an increase in the number of receptors (see IL 104355 and EL 109633). Corresponding studies concerning the control of FAS-R at the level of the promoter of the FAS-R gene have yet to be reported.

Brief Summary Text (16):

Further, it should also be mentioned that, while it is known that the tumor necrosis factor (TNF) receptors, and the structurally-related receptor FAS-R, trigger in cells, upon stimulation by leukocyte-produced ligands, destructive activities that lead to their own demise, the mechanisms of this triggering are still little understood. Mutational studies indicate that in FAS-R and the p55 TNF receptor (p55-R) signaling for cytotoxicity involve distinct regions within their intracellular domains (Brakebusch et al., 1992; Tartaglia et al., 1993; Itoh and Nagata, 1993). These regions (the `death domains`) have sequence similarity. The `death domains` of both FAS-R and p55-R tend to self-associate. Their self-association apparently promotes that receptor aggregation which is necessary for initiation of signaling (see IL 109632, IL 111125 and IL 1 12002, as well as Song et al., 1994; Wallach et al., 1994; Boldin et al., 1995) and at high levels of receptor expression can result in triggering of ligand-independent signaling (IL 109632, IL 111125 and Boldin et al., 1995).

Brief Summary Text (18):
It should be noted however, as is set forth hereinbelow, that in accordance with the present invention, it has been discovered that the upper part of the above noted regulatory (C-terminal) domain of ankyrin contains a so-called `death domain` motif, which may function to mediate the binding of proteins together (activity of the first two ankyrin domains), or may function conformationally to regulate the arkyrin protein.

Brief Summary Text (20):
It should be noted however, as is set forth hereinbelow, that in accordance with the present invention, it has been discovered that the NGF-R contains a `death domain` motif in its intracellular domain, which may be involved in the mediation of the intracellular events associated with the regulatory role played by NGF-R with regards to cell viability.

Brief Summary Text (21):
MORT-1 is a recently discovered protein that binds to the intracellular domain of FAS-R, is capable of self-association and can activate cell cytotoxicity on its own. Hence, MORT1 is also a regulatory protein involved in intracellular signaling processes. It was also discovered that MORT-1 has a `death domain` motif that is associated with its observed biological activity (see co-pending IL 112002 and IL 112692).

Brief Summary Text (22):
Two further intracellular proteins, RIP (Stanger et al., 1995) and TRADD (Hsu et al., 1995), that bind to the intracellular domains of p55 TNF-R or FAS-R and apparently take part in the induction of their cytocidal effect, have recently been cloned. All three proteins, MORT-1, RIP and TRADD, were found to contain the sequence motif shared between the `death domains` of the intracellular domains of p55-TNF-R and FAS-R. As in the receptors, the `death domain` motifs (DD) in the three intraceuular proteins seem to be sites of protein-protein interaction. The three proteins interact with the p55-TNF-R and FAS-R intracellular domains by the binding of their DDs to those in the receptors, and in both TRADD and RIP (though not in MORT-1) the DDs self-associate. It has now been found that MORT-1 and TRADD bind differentially to FAS-R and p55 TNF-R and also bind to each other. Moreover, both bind effectively to RIP.

Brief Summary Text (23):
Interference of the interaction between the above three intracellular proteins will result in modulation of the effects caused by this interaction. Thus, inhibition of TRADD binding to MORT-1 may modulate FAS-R-p55 TNF-R intraction. Inhibition of RIP in addition to the above inhibition of TRADD binding to MORT-1 may further modulate FAS-R-p55 TNF-R interaction.

Brief Summary Text (24):
Monoclonal antibodies raised against the `death domain` of the p55 TNF-R, specifically against the binding site or sites of TRADD and RIP can also be used to inhibit or prevent binding of these proteins and thus cause modulation of the interaction between the FAS-R and the p55 TNF-R.

Brief Summary Text (25):
In a way analogous to that noted above in respect of TNFJNF-R and FAS-ligand/FAS-R, there is also a need to provide a way for modulating the activity of the above noted proteins, i.e. ankyrin, NGF-R and MORT-1, namely, to inhibit their activity when it is associated with detrimental effects, e.g. disease/disorder-related cell cytotoxicity or conformational changes in cell-shape; or to enhance their activity when this is desired, e.g. for directed destruction of diseased cells, etc.

Brief Summary Text (27):
The present invention concerns modulators such as proteins, peptides, antibodies and organic compounds which are capable of interacting/binding with one or more so-called `death domain` motifs in the intracellular domains of proteins containing such motifs, these proteins being related, e.g. members of the TNF/NGF receptor family or proteins related thereto, e.g. MORT1, or unrelated proteins, e.g. ankyrins. These modulators are characterized by recognizing general structural features common to the `death domain` motifs of the `death domain` motif-containing proteins, and by also recognizing specific structural features present in each of the different `death domain` motifs of these proteins.

Brief Summary Text (28):
Accordingly, it is one aim of the invention to provide modulators, as noted above, capable of binding to or interacting with the `death domain` motifs of one or more of the `death domain` motif-containing proteins and thereby modulating the activity of these proteins.

Brief Summary Text (29):
Another aim of the invention is to provide antagonists (e.g. antibodies) to one class of these modulators, namely the naturally-occurring proteins or peptides which bind to `death domain` motif-containing proteins, and which antagonists may be used to inhibit the signaling process, when desired, when such `death domain` motif-binding proteins or peptides are positive signal effectors (i.e. induce signaling), or to enhance the signaling process, when desired, when such `death domain` motif-binding proteins are negative signal effectors (i.e. inhibit signaling).

Brief Summary Text (30):
Yet another aim of the invention is to use such `death domain` motif-binding proteins or peptides to isolate and characterize additional proteins or factors, which may, for example, be involved further downstream in the signaling process, and/or to isolate and identify other receptors further upstream in the signaling process to which these `death domain` motif-binding proteins bind, and hence, in whose function they are also involved.

Brief Summary Text (31):
Moreover, it is an aim of the present invention to use the above-mentioned `death domain` motif-binding proteins as antigens for the preparation of polyclonal and/or monoclonal antibodies thereto. The antibodies. in turn, may be used for the purification of the new `death domain` motif-binding proteins from different sources, such as cell extracts or transformed cell lines.

Brief Summary Text (32):
Furthermore, these antibodies may be used for diagnostic purposes, e.g. for identifying disorders related to abnormal functioning of cellular effects mediated by the various proteins belonging to the group of `death domain` motif-containing proteins.

Brief Summary Text (33):
A further aim of the invention is to provide pharmaceutical compositions comprising the above `death domain` motif-binding modulators (proteins, peptides, organic molecules), and pharmaceutical compositions comprising the `death domain` motif-binding protein or peptide antagonists, for the treatment or prophylaxis of conditions related to the activity of the `death domain` motif-containing proteins, for example, such compositions can be used to enhance the TNF or FAS ligand effect or effects mediated by NGF-R, MORT-1, RIP, TRADD and ank-yrin, or to inhibit the TNF or FAS ligand effect or effects mediated by depending on the above noted nature of the `death domain` motif-binding modulators or antagonists thereof contained in the composition.

Brief Summary Text (34):
A still further aim of the invention is to use the various `death domain` motifs of the proteins containing them for the design and synthesis of complementary peptides and organic molecules which will be modulators of these proteins.

Brief Summary Text (36):
The present invention is based on the surprising and unexpected finding that there exists a so-called `death domain` motif in a wide range of proteins some of which are related and others which are not related. For example, this `death domain` motif has been found in p55 TNF-R, FAS-R, NGF-R, MORTI, RIP and TRADD which are related to each other, as well as in the unrelated protein, ankyrin 1.

Brief Summary Text (37):
As noted above, the `death domain` motif of the proteins containing this motif is located in the intracellular regulatory domain of these proteins. Hence, the `death domain` motif appears to be involved in a regulatory function associated with cell viability (cell death) as well as cell shape/conformation, this function being effected at (i.e. in the case of receptors containing this motif) or close to (i.e. in the case of structural intracellular proteins, e.g. ankyrin) the cell surface. Moreover, the observation, in accordance with the present invention, that the `death domain` motif is conserved amongst a wide range of related and non-related proteins indicates that this motif may have an important regulatory function.

Brief Summary Text (38):
Accordingly, the present invention provides a modulator of regulatory cellular events occurring intracellularly that are mediated by regulatory proteins containing a `death domain` motif which is a regulatory portion of said proteins, said modulator being capable of interacting with one or more of the `death domain` motifs contained in said regulatory proteins and affecting the regulatory action of one or more of said regulatory proteins.

Brief Summary Text (40):

(i) a modulator is selected from the group comprising naturally-derived `death domain` motif-binding proteins and peptides and analogs and derivatives thereof capable of interacting with one or more of said `death domain` motifs;

Brief Summary Text (41):
(ii) a modulator is selected from the group of synthetically produced complementary peptides, synthesized by using as substrates the `death domain` motif sequences of said regulatory proteins containing `death domain` motifs, said complementary peptides being capable of interacting with one or more of said `death domain` motifs.

Brief Summary Text (42):
(iii) a modulator is selected from the group comprising antibodies or active fragments thereof capable of interacting with one or more of said `death domain` motifs.

Brief Summary Text (43):
(iv) a modulator is selected from the group of organic compounds capable of interacting with one or more of said `death domain` motifs, said organic compounds being derived from known compounds and selected by using said `death domain` motifs as a substrate in a binding assay, or being synthesized using said `death domain` motifs as a substrate for designing and synthesizing said organic compounds.

Brief Summary Text (44):
(v) a modulator is selected from the group of peptides or polypeptides derived from naturally occurring `death domain` motif sequences, said peptides or polypeptides being capable of interacting with one or more of said `death domain` motifs, and analogs and derivatives of said peptides or polypeptides capable of interacting with one or more of said `death domain` motifs.

Brief Summary Text (45):
(vi) a modulator of any one of (i)-(v) wherein said modulator is further characterized by being capable of recognizing the general `death domain` motif sequence features common to the `death domain` motifs of `death domain` motif containing proteins, and being capable of recognizing one or more of the specific `death domain` motifs of said proteins, said specific sequence features being specific to each `death domain` motif sequence of each of said proteins.

Brief Summary Text (46):
(vii) a modulator of any one of (i)-(vi) wherein said modulator is capable of interacting with one or more of the `death domain` motifs contained within the proteins belonging to the group comprising p55 TNF-R, FAS-R, NGF-R, MORT-1, RIP, TRADD and ankyrin 1.

Brief Summary Text (47):
(viii) a modulator of (vii) wherein said modulator is further characterized by being capable of interacting with common sequence features of the `death domain` motifs of said group of proteins, said common sequence features comprising the group of common amino acid residues W (tryptophan), L (leucine), I (isoleucine), A (alanine), D (aspartic acid), E (glutamic acid), T (threonine), R (arginine) and Y (tyrosine) at the location within said `death domain` motifs shown in FIG. 1.

Brief Summary Text (50):
(a) a cDNA sequence derived from the coding region of a native `death domain` motif-binding protein or peptide,

Brief Summary Text (51):
(b) DNA sequences capable of hybridization to a sequence of (a) under moderately stringent conditions and which encode a biologically active `death domain` motif-binding protein or peptide; and

Brief Summary Text (52):
(c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which encode a biologically active `death domain` motif-binding protein or peptide.

Brief Summary Text (54):
(i) DNA sequence encoding a `death domain` motif-binding protein or peptide capable of binding to the `death domain` motif of one or more of the proteins of the group comprising p55 TNF-R, FAS-R, NGF-R, MORT-1 and anlvrin 1.

Brief Summary Text (55):
(ii) DNA sequence encoding a peptide or polypeptide derived from the naturally occurring `death domain` motif sequence of the `death domain` motif-containing proteins.

Brief Summary Text (56):
(iii) DNA sequence encoding a peptide or polypeptide derived from the `death domain` motif sequence of any one of the proteins

of the group comprising p55 TNF-R, FAS-R, NGF-R, MORT-1, RIP, TRADD and ankyrin 1.

Brief Summary Text (58):
(a) a protein, peptide or polypeptide and analogs of any one thereof encoded by a DNA sequence of the invention. said protein, peptide, polypeptide and analogs being capable of binding to or interacting with one or more of the `death domain` motifs of one or more `death domain` motif containing proteins.

Brief Summary Text (65):
The present invention also provides a method for the modulation of the TNF or FAS-R ligand effect on cells mediated by p55 TNF-R and FAS-R, or the functions mediated in cells by NGF-R, MORT-1, RIP, TRADD, ankyrin 1 or by other proteins containing a `death domain` motif, comprising treating said cells with one or more proteins, peptides, polypeptides or analogs selected from the group consisting of the proteins, peptides, polypeptides or analogs of the invention (see (a) above), all being capable of binding to or interacting with the `death domain` motif and modulating the activity of said `death domain` motif-containing proteins, wherein said treating of said cells comprises introducing into said cells said one or more proteins, peptides, polypeptides or analogs in a form suitable for intracellular introduction thereof, or introducing into said cells a DNA sequence encoding said one or more proteins, peptides, polypeptides or analogs in the form of a suitable vector carrying said sequence, said vector being capable of effecting the insertion of said sequence into said cells in a way that said sequence is expressed in said cells.

Brief Summary Text (67):
(a) constructing a recombinant animal virus vector carrying a sequence encoding a viral surface protein (ligand) that is capable of binding to a specific cell surface receptor on the surface of said cell to be treated and a second sequence encoding a protein selected from the proteins, peptides, polypeptides and analogs of the invention, said protein, peptide, polypeptide or analogs, when expressed in said cells being capable of modulating the activity of said `death domain` motif-containing protein; and

Brief Summary Text (69):
Another method of the invention is a method for modulating the TNF or FAS-R ligand effect on cells mediated by p55 TNF-R and FAS-R, or the functions mediated in cells by NGF-R, MORT-1,RIP, TRADD, ankyrin 1 or by other proteins containing a `death domain` motif, comprising treating said cells with antibodies or active fragments or derivatives thereof, of the invention (see (g) above), said treating being by application of a suitable composition containing said antibodies, active fragments or derivatives thereof to said cells, said composition being formulated for intracellular application.

Brief Summary Text (70):
Yet another method of the invention is a method for modulating the TNF or FAS-R ligand effect on cells mediated by p55 TNF-R and FAS-R, or the functions mediated in cells by NGF-R, MORT-1, RIP, TRADD, ankyrin 1 or by other proteins containing a `death domain` motif, comprising treating said cells with an oligonucleotide sequence selected from a sequence encoding an antisense sequence of at least part of the sequence of the invention as noted above, said oligonucleotide sequence being capable of blocking the expression of at least one of the `death domain` motif-binding proteins or peptides.

Brief Summary Text (76):
(ii) a method for modulating the TNF or FAS-R ligand effect on cells mediaed by p55 TNF-R and FAS-R, or the fuinctions mediated in cells by NGF-R, MORT-1, RIP, TRADD, ankyrin 1 or by other proteins containing a `death domain` motif, comprising applying the ribozyme procedure in which a vector encoding a ribozyme sequence capable of interacting with a cellular mRNA sequence encoding a protein or peptide of the invention, is introduced into said cells in a form that permits expression of said ribozyne sequence in said cells, and wherein when said ribozyme sequence is expressed in said cells it interacts with said cellular mRNA sequence and cleaves said mRNA sequence resulting in the inhibition of expression of said protein or peptide in said cells.

Brief Summary Text (77):
(iii) a method for isolating and identifying proteins, peptides, factors or receptors capable of binding to the `death domain` motif-binding proteins or peptides of the invention, comprising applying the procedure of affinity chromatography in which said protein or peptide of the invention is attached to the affinity chromatography matrix, said attached protein is brought into contact with a cell extract and proteins, factors or receptors from cell extract which bound to said attached protein are then eluted, isolated analyzed.

Brief Summary Text (78):
(iv) a method for isolating and identifying proteins, capable of binding to the `death domain` motif-binding proteins or peptides of the invention, comprising applying the yeast two-hybrid procedure in which a sequence encoding said `death domain` motif-binding protein is carried by one hybrid vector and sequence from a cDNA or genomic DNA library are carried by the second hybrid vector, the vectors then being used to transform yeast host cells and the positive transformed cells being isolated, followed by extraction of the said second hybrid vector to obtain a sequence encoding a protein which binds to said `death

domain` motif-binding protein.

Brief Summary Text (79):
The present invention also provides a pharmaceutical composition for the modulation of the TNF- or FAS-R ligand- effect on cells mediated by p5.sup.5 TNF-R and FAS-R, or the fuinctions mediated in cells by NGF-R, MORT-1, RIP, TRADD, ankyrin 1 or by other proteins containing a `death domain` motif comprising, as active, ingredient a modulator of the invention.

Brief Summary Text (81):
(i) a pharmaceutical composition for modulating the TNF- or FAS-R ligand-effect on cells mediated by p55 TNF-R and FAS-R, or the functions mediated in cells by NGF-R, MORT-1, RIP, TRADD, ankyrin 1 or by other proteins containing a `death domain` motif, comprising, as active ingredient, a recombinant animal virus vector encoding a protein capable of binding a cell surface receptor and encoding a protein or peptide or analogs thereof of the invention.

Brief Summary Text (82):
(ii) a pharmaceutical composition for modulating the TNF or FAS-R ligand effect on cells mediated by p55 TNF-R and FAS-R, or the functions mediated in cells by NGF-R, MORT-1, RIP, TRADD, ankyrin 1 or by other proteins containing a `death domain` motif, comprising as active ingredient, an oligonucleotide sequence encoding an anti-sense sequence of the sequence of the invention.

Brief Summary Text (83):
A still further method of the invention is a method for isolating and identifying a protein capable of binding to the `death domain` motifs of `death domain` motif-containing proteins comprising applying the procedure of non-stringent southern hybridization followed by PCR cloning, in which a sequence or parts thereof of the invention is used as a probe to bind sequences from a cDNA or genomic DNA library, having at least partial homology thereto, said bound sequences then amplified and cloned by the PCR procedure to yield clones encoding proteins having at least partial; homology to said sequences of the invention.

Brief Summary Text (84):
In addition, the present invention also provides a method for designing drugs that are capable of modulating the activity of `death domain` motif-containing proteins, comprising the procedures described herein in Examples 3 and 4.

Brief Summary Text (86):
It should be noted that, where used throughout, the following terms "Modulation/Mediation of the TNF or FAS-R ligand effect on cells mediated by p55 TNF-R and FAS-R, or the flunctions mediated in cells by NGF-R, MORT1, RIP, TRADD, ankyrin 1 or by other proteins containing a `death domain` motif are understood to encompass in vitro as well as in vivo treatment.

Drawing Description Text (2):
FIG. 1 depicts schematically the sequence homology of the `death domain` motif in MORT-1(SEQ ID NO:5), p55 TNF-R (SEQ ID NO:3), Fas/APO1 (FAS-R) (SEQ ID NO:1), low affinity NGF receptor (NGF-R) (SEQ ID NO:4) and the C-terminal part of the regulatory domain in ankyrin 1 (Ankyrin 1) (SEQ ID NO:2), as described in Example 1.

Drawing Description Text (3):
FIG. 2 depicts interactions of the `death domains` of the p55-R, Fas/APOl, MORT1, TRADD and RIP in a yeast two-hybrid test, and the effect of lpr.sup.cg -like mutations in these proteins on their interactions. Assessment of the interaction of Gal4 hybrid constructs encompassing the following human proteins, trunctated upstream to their DD motifs: p55-R (residues 326-426), FAS-R (residues 210-319), MORT-1 (residues 92-208), TRADD (residues 195-312) and RIP (residues 261-372), as well as of the following points mutants of these proteins: p55-R L35IN, FAS-R V238N, MORT-1 V121N, and RIP F308N, whose mutation sites within the DDs correspond to that found in the FAS-R of the Ipr.sup.cg mice. Each cDNA insert was introduced both into the Gal4 DNA binding domain (DBD) and the Gal4 activation domain (AD) constructs (pGBT9 abd pGAD-GH), and the binding of the inserts in both constructs to all other inserts within transfected SFY526 yeasts was assessed by a .beta.-galactosidase expression filter assay. The results are presented in terms of the time required for development of strong color. ND--not done.

Detailed Description Text (2):
The present invention relates, in one aspect, to novel proteins or peptides which are capable of binding to one or more `death domain` motifs of `death domain` motif-containing proteins by virtue of recognizing sequence features common to the `death domain` motifs within these proteins. Hence the `death domain` motif binding proteins or peptides are considered as mediators or modulators of this group of `death domain` motif-containing proteins. This group of `death domain` motif-containing proteins includes: (i) members of the TNF/NGF receptor family such as, for example, p55 TNF-R, FAS-R (Fas/APO1) and the low affinity NGF receptor (NGF-R); (ii) other related proteins such as, for example, the recently discovered protein called MORT-1 (or HF1) (for "Mediator of Receptor-Mediated Toxicity") which, amongst its characteristics, is capable of self-association and

specific binding to the intracellular domain of FAS-R; as well as (iii) apparently non-related proteins such as, for example, the cytoskeletal protein ankyrin 1. The `death domain` motif and some of its characteristics has been disclosed in respect of the p55 TNF-R, FAS-R and MORT-1 in the co-pending Israel Application Nos. 109632, 111125, 112002 and 112692. The `death domain` motif present in NGF-R and ankyrin 1 has been discovered in accordance with the present invention (see Example 1).

Detailed Description Text (3):
In the above noted co-pending applications there is described a number of proteins capable of binding specifically to the intracellular domains of p55-TNF-R and/or FAS-R, which proteins include MORT-1. However, in contrast, the present invention concerns, in this one aspect thereof, proteins or peptides which specifically bind to the `death domain` motif of one or more of the above mentioned proteins belonging to the group characterized by having such a `death domain` motif, the binding/interaction between the proteins or peptides of the invention and the `death domain` motif being by virtue of sequence features common to the various `death domain` motifs. Hence, the proteins or peptides of the invention are characterized by being capable of modulating or mediating the activity of one or more of the members of this group of proteins by recognizing features common to the `death domain` motifs.

Detailed Description Text (4):
Accordingly, included in the present invention is a large group of proteins or peptides which bind to the various `death domain` motifs, in which some of the proteins or peptides bind specific `death domain` motifs of specific proteins or receptors, while others bind more than one such motif of more than one such protein/receptor. From FIG. 1 it arises that common sequence features of the `death domain` motifs in `death domain` motif-containing proteins such as p55 TNF-R, FAS-R, NGF-R, MORT1 and ankyrin 1 include common amino acid residues (residues marked within boxes) such as the W (tryptophan), L (leucine), I (isoleucine), A (alanine), D (aspartic acid) and E (glutamic acid), as well as T (threonine), R (arginine) and Y (tyrosine), at the location shown in FIG. 1.

Detailed Description Text (5):
The proteins or peptides of the invention may be obtained as described in the above noted co-pending patent applications (see also Example 3), by use of the yeast two-hybrid procedure in which the `death domain` motif of, for example, p55-TNF-R, FAS-R, MORT-1, NGF-R, ankyrin 1 will be used as probes or 'baits' to isolate from genomic or cDNA libraries, clones expressing proteins or peptides capable of binding to one or more of these `death domain` motifs. Alternatively, a synthetic DNA sequence can be synthesized in which there is included all of the common sequence features of the `death domain` motifs of p55-TNF-R, FAS-R, MORT-1, NGF-R, ankyrin 1 (see FIG. 1), to provide a common or "universal" `death domain` motif sequence. which in turn can be used in the yeast two-hybrid procedure to isolate and identify clones from cDNA or genoriic libraries which encode proteins or peptides capable of binding to this `death domain` motif sequence.

Detailed Description Text (6):
Other approaches for obtaining the proteins and peptides of the invention include the well known standard procedures such as, for example, affinity chromatography in which, for example, peptides or protein fragments having the `death domain` motif sequence of p55 TNF-R, FAS-R, MORTI, NGF-R and ankyrin 1; or a synthetically produced `death domain` motif peptide having common sequence features of all the aforesaid `death domain` motifs (see FIG. 1), are attached to the chromatography substrate or matrix and are brought into contact with cell extracts or lysates (of human/mammalian origin) and thereby proteins or peptides are isolated which are capable of binding to one or more of these `death domain` motifs. Likewise, other standard chemical and recombinant DNA procedures usually employed for isolating proteins or peptides capable of binding to a specific amino acid sequence (`death domain` motif sequence) can be employed to obtain the proteins and peptides of the invention.

Detailed Description Text (8):
Moreover, the present invention also concerns the DNA sequences encoding biologically active analogs and derivatives of these proteins and peptides of the invention, and the analogs and derivatives encoded thereby. The preparation of such analogs and derivatives is by standard procedure (see for example, Sambrook et al., 1989) in which in the DNA sequences encoding these proteins, one or more codons may be deleted, added or substituted by another, to yield analogs having at least a one amino acid residue change with respect to the native protein. Acceptable analogs are those which retain at least the capability of binding to the `death domain` motif of one or more of the members of the above mentioned group of `death domain` motif-containing proteins, or which can mediate any other binding or enzymatic activity, e.g. analogs which bind the `death domain` motif but which do not signal, i.e. do not bind to a further downstream receptor, protein or other factor, or do not catalyze a signal-dependent reaction. In such a way analogs can be produced which have a so-called dominant-negative effect, namely, an analog which is defective either in binding to the, `death domain` motif or in subsequent signaling following such binding. Such analogs can be used, for example, to inhibit the TNF, FAS-ligand-, NGF-R-mediated, MORT-1-mediated and ankyrin 1-mediated effect by competing with the natural IC-binding proteins.

Detailed Description Text (9):
Likewise, so-called dominant-positive analogs may be produced which would serve to enhance, for example, the TNF, FAS ligand. NGF-R-mediated, MORT-1-mediated and ankyrin 1- mediated effect. These would have the same or better `death

domain` motif-binding properties and the same or better signaling properties of the natural `death domain` motif-binding proteins. Similarly, derivatives may be prepared by standard modifications of the side groups of one or more amino acid residues of the proteins, or by conjugation of the proteins to another molecule e.g. an antibody, enzyme, receptor, etc., as are well known in the art.

Detailed Description Text (10):
The new `death domain` motif-binding proteins and peptides of the invention, e.g. the proteins and peptides capable of binding one or more of the `death domain` motifs of p55 TNF-R, FAS-R, MORT-1, NGF-R and ankyrin 1, as well as RIP and TRADD, have a number of possible uses, for example:

Detailed Description Text (11):
(i) They may be used to mimic or enhance the function of TNF or FAS-R ligand, or the functions mediated by NGF-R, MORT-1, RIP, TRADD and ankyrin 1 or other proteins containing the `death domain` motif, in situations where such an enhanced effect is desired such as in anti-tumor, anti-inflammatory, or anti-HIV or other disease/disorder applications where the enhanced activity is desired. In this case the proteins or peptides may be introduced to the cells by standard procedures known per se. For example, as the proteins or peptides are required to act intracellularly, i.e. bind/interact with intracellularly located `death domain` motifs and it is desired that they be introduced only into the cells where their effect is wanted, a system for specific introduction of these proteins into the cells is necessary. One way of doing this is by creating a recombinant animal virus e.g. one derived from Vaccinia, to the DNA of which the following two genes will be introduced: the gene encoding a ligand that binds to cell surface proteins specifically expressed by the cells e.g. ones such as the AIDs (HIV) virus gp120 protein which binds specifically to some cells (CD4 lymphocytes and related leukemias), or a ligand that binds specifically to erythrocytes or nervous tissue (in the case of ankyrin 1), or a ligand binding specifically to cells characterized by expressing other members of the `death domain` motif-containing group of proteins, e.g. those expressing MORT-1, RIP, TRADD, or any other ligand that binds specifically to cells carrying a TNF-R, FAS-R, or NGF-R such that the recombinant virus vector will be capable of binding such cells; and the gene encoding the new `death domain` motif-binding protein or peptide. Thus, expression of the cell-surface-binding protein on the surface of the virus will target the virus specifically to the tumor cell, HIV-infected cells or other cells, following which the `death domain` motif-binding protein or peptide encoding sequence will be introduced into the cells via the virus, and once expressed in the cells will result in enhancement of, for example, the TNF, FAS-R ligand, NGF-R-mediated, MORT-1-mediated, RIP- and TRADD-mediated, or ankyrin 1-mediated effect leading to, for example, the death of the tumor cells or other TNF-R- or FAS-R- carrying cells it is desired to kill. Construction of such recombinant animal virus is by standard procedures (see for example, Sambrook et al., 1989). Another possibility is to introduce the sequences of the new proteins or peptides in the form of oligonucleotides which can be absorbed by the cells and expressed therein.

Detailed Description Text (12):
(ii) They may be used to inhibit, for example, the TNF, FAS-R ligand, NGF-R-mediated, MORT1-mediated and aknyrin-l-mediated effect, e.g. in cases such as tissue damage in septic shock, graft-vs.-host rejection, acute hepatitis, or other diseases/disorders in which case it is desired to block the TNF-induced TNF-R, FAS-R ligand induced FAS-R or NGF induced NGF-R intracellular signaling or intracellular events mediated by MORT1, RIP, TRADD and ankyrin-1. In this situation it is possible, for example, to introduce into the cells, by standard procedures, oligonucleotides having the, anti-sense coding sequence for these new proteins or peptides which would effectively block the translation of mRNAs encoding these proteins and thereby block their expression and lead to the above noted desired inhibition of the effects mediated by the `death domain` motif-containing proteins.

Detailed Description Text (13):
Such oligonucleotides may be introduced into the cells using the above recombinant virus approach, the second sequence carried by the virus being the oligonucleotide sequence. Another possibility is to use antibodies specific for these proteins or peptides to inhibit their intracellular signaling activity (via their binding to the `death domain` motifs).

Detailed Description Text (14):
Yet another way of inhibiting the TNF FAS-R ligand, NGF-R-mediated, MORT-1-mediated, RIP- and TRADD-mediated, or ankyrin-1-mediated effect or effects mediated by other `death domain` motif-containing proteins, is by the recently developed ribozyme approach. Ribozymes are catalytic RNA molecules that specifically cleave RNAs. Ribozymes may be engineered to cleave target RNAs of choice, e.g. the mRNAs encoding the new proteins or peptides of the invention. Such ribozymes would have a sequence specific for the mRNA of choice and would be capable of interacting therewith (complementary binding) followed by cleavage of the MRNA, resulting in a decrease (or complete loss) in the expression of the protein or peptide it is desired to inhibit, the level of decreased expression being dependent upon the level of ribozyme expression in the target cell. To introduce ribozymes into the cells of choice any suitable vector may be used, e.g. plasmid, animal virus (retrovirus) vectors, that are usually used for this purpose (see also (i) above, where the virus has, as second sequence, a cDNA encoding the ribozyme sequence of choice). Moreover, ribozymes can be constructed which have multiple targets (multi-target ribozymes) that can be used, for example, to inhibit the expression of one or more of the proteins or peptides of the invention (For reviews, methods etc. concerning ribozymes see Chen et al., 1992; Zhao and Pick, 1993; Shore et al., 1993; Joseph and Burke, 1993; Shimayama et al.,

1993; Cantor et al., 1993; Barinaga, 1993; Crisell et al., 1993 and Koizumi et al., 1993).

Detailed Description Text (15):
(iii) They may be used to isolate, identify and clone other proteins or peptides which are capable of binding to them, e.g. other proteins or peptides involved in the intracellular signaling process that are downstream of the `death domain` motif-containing proteins. In this situation, these options, namely, the DNA sequences encoding them may be used in the yeast two-hybrid system (see Example 2, below) in which the sequence of these proteins or peptides will be used as "baits" to isolate, clone and identify from cDNA or genomic DNA libraries other sequences ("preys") encoding proteins which can bind to these new `death domain` motif-binding proteins. In the same way, it may also be determined whether the specific proteins or peptides of the present invention, namely, those which bind to the `death domain` motif of p55 TNF-R, FAS-R, NGF-R, MORT-1 and ankyrin can bind to yet other receptors or proteins. Moreover, this approach may also be taken to determine whether the proteins or peptides of the present invention are capable of binding to other known receptors or proteins in whose activity they may have a functional role, i.e. other aas yet unidentified `death domain` motif-containing receptors or proteins.

Detailed Description Text (16):
(iv) The new proteins may also be used to isolate, identify and clone other proteins of the same class i.e. those binding to `death domain` motifs of the various receptors or proteins listed above or to functionally related receptors or proteins, and involved in their modulation/mediation. In this application the above noted yeast two-hybrid system may be used, or there may be used a recently developed (Wilks et al., 1989) system employing non-stringent southern hybridization followed by PCR cloning. In the Wilks et al. publication, there is described the identification and cloning of two putative protein-tyrosine kinases by application of non-stringent southern hybridization followed by cloning by PCR based on the known sequence of the kinase motif, a conceived kinase sequence. This approach may be used, in accordance with the present invention using the sequences of the new proteins or peptides to identify and clone those of related `death domain` motif-binding proteins or peptides also capable of binding to `death domain` motif-containing receptors or proteins.

Detailed Description Text (18):
(vi) As noted above, the new proteins or peptides of the invention may also be used as inmmunogens (antigens) to produce specific antibodies thereto. These antibodies may also be used for the purposes of purification of the new proteins or peptides either from cell extracts or from transformed cell lines producing them. Further, these antibodies may be used for diagnostic purposes for identifying disorders related to abnormal functioning of, for example, the TNF, FAS-R ligand, NGF-R, MORT-1 or ankyrin 1 system, e.g. overactive or underactive TNF- or FAS-R ligand-induced cellular effect or NGF-R-, MORT-1- or ankyrin-1 mediated cellular effects. Thus, should such disorders be related to a malfunctioning intracellular signaling or structural regulation system involving the new proteins or antibodies, such antibodies would serve as an important diagnostic tool.

Detailed Description Text (19):
In another aspect, the present invention relates to complementary peptides which may be synthesized by well known standard procedures of the art, that are capable of binding or interacting specifically with one or more of the `death domain` motifs of the above mentioned group of `death domain` motif-containing proteins. These complementary peptides will be synthesized using, for example, the `death domain` motif sequences of p55-TNF-R, FAS-R, MORT-1, RIP, TRADD, NGF-R, ankyrin 1, as substrates and synthesizing by standard chemical means peptides of sequence that are complementary to these `death domain` motif sequences. A suitable complementary peptide is one that will be capable of binding to one or more of these `death domain` motifs and thereby being capable of modulating or mediating the activity of `death domain` motif-containing proteins.

Detailed Description Text (20):
The complementary peptides may be generated using as substrate one or more of the `death domain` motif sequences set forth in FIG. 1 or may be generated using a synthetic peptide (see above) which has a sequence inclusive of all of the common sequence features of the known `death domain` motif sequences, e.g. the above mentioned amino acid residues W, L, I, A, D, E, T, R and Y.

Detailed Description Text (21):
The so-generated complementary peptides, and likewise, DNA sequences encoding them, which may be readily produced by standard procedures, may be employed, as noted above in any one of uses (i)-(vi), i.e. to enhance (gain-of-function) or inhibit the activity of proteins or receptors containing a `death domain` motif, or may be used to generate specific antibodies thereto for modulation/mediation, isolation or diagnostic purposes.

Detailed Description Text (22):
It should also be noted that included in the present invention are the antibodies (and their uses) specific to the proteins and peptides of the invention including the complementary peptides, as well as antibodies specific to the `death domain` motif peptides themselves, e.g. those peptides shown in FIG. 1 which are the `death domain` motifs of p55-TNF-R, FAS-R, MORT-1, NGF-R, ankyrin 1 and other proteins containing the `death domain` motif. These antibodies may be used for directly

modulating/mediating the activity of proteins or receptors containing `death domain` motifs or for isolation, identification and characterization (including diagnostic applications, as noted above) of other proteins and receptors containing such `death domain` motifs.

Detailed Description Text (28):
Accordingly, mAbs generated against the `death domain` motif-containing peptides, `death domain` -binding proteins or peptides, or `death domain` -binding complementary peptides, analogs or derivatives thereof of the invention may be used to induce anti-Id antibodies in suitable animals, such as BALB/c mice. Spleen cells from such immunized mice are used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for an epitope of the above proteins, peptides, analogs or derivatives.

Detailed Description Text (31):
It will be appreciated that Fab and F(ab').sub.2 and other fragments of the antibodies useful in the present invention may be used for the detection and quantitation of the `death-domain`-binding proteins or peptides according to the methods disclosed herein for intact antibody molecules. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab').sub.2 fragments).

Detailed Description Text (34):
The antibodies, including fragments of antibodies, useful in the present invention may be used to quantitatively or qualitatively detect the `death domain` motif-binding proteins or peptides (including complementary peptides) in a sample or to detect presence of cells which express the `death domain` motif-binding proteins or peptides of the present invention. This can be accomplished by immunofluorescence techniques employing a fluorescently labeled antibody (see below) coupled with light microscopic, flow cytometric, or fluorometric detection.

Detailed Description Text (35):
The antibodies (or fragments thereof) useful in the present invention may be employed histologically, as in immunofluorescence or immunoelectron microscopy, for in situ detection of `death domain` motif-binding proteins or peptides of the present invention. In situ detection may be accomplished by removing a histological specimen from a patient, and providing the labeled antibody of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the `death domain` motif-binding proteins or peptides, but also its distribution on the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of wide variety of histological methods (such as staining procedures) can be modified in order to achieve such in sits detection.

Detailed Description Text (36):
Such assays for `death domain` motif-binding proteins of the present invention typically comprises incubating a biological sample, such as a biological fluid, a tissue extract, freshly harvested cells such as lymphocytes or leukocytes, or cells which have been incubated in tissue culture, in the presence of a detectably labeled antibody capable of identifying the `death domain` motif-binding proteins or peptides, and detecting the antibody by any of a number of techniques well known in the art.

Detailed Description Text (52):
In another aspect, the present invention relates to the use of the various different `death domain` motifs or the synthetically produced "universal" `death domain` motif (having structural features common to many different `death domain` motifs) as agents for enhancing (gain of function) the intracellular effect mediated by the natural `death domain` motif-containing proteins. In this aspect the `death domain` motifs will be used in the form of peptides containing all of the `death domain` motif or active parts thereof and introduced into the cells as mentioned above (e.g. the vaccinia virus approach). In this regard it should be noted that the term `death domain` was coined following the discovery (see the co-pending patent applications noted above) that this region of the intracellular domains of the p55 TNF-R and FAS-R was the region involved in the ligand-independent self-association and cell-cytotoxicity induction mediated by these receptors. In fact, the free `death domain` of p55 TNF-R (p55DD) is capable of self-associating and inducing cell cytotoxicity. Further, upon discovery of the MORT1 protein which is a FAS-R binding protein, it was also found that this protein is capable of self-association and inducing, in a ligand-independent and FAS-R-independent manner, cytotoxic effects on cells. The MORT-1 protein was subsequently observed to contain a `death domain` motif homologous to the `death domains` or `death domain` motifs of p55 TNF-R and FAS-R (see FIG. 1), which `death domain` motif is involved in MORT1 association with FAS-R and is associated with the MORT1 protein's ability to induce cell cytotoxic effects.

Detailed Description Text (53):
Thus, using the `death domain` motifs of proteins such as p55-TNF-R, FAS-R and MORT1 and any other proteins involved in the induction of cytotoxic effects, in the way described above, it is possible to enhance the cell cytotoxic effects normally mediated by the naturally-occuring counterparts of these proteins, i.e. it would be possible to enhance the killing of cells such as

tumor cells, HTV-infected and other diseased cells, the killing of which is usually mediated by p55 TNF-R, FAS-R, MORT1, RIP or TRADD, by introducing into such cells the `death domain` motifs of these receptors/proteins.

Detailed Description Text (54):
Moreover, it is also possible to produce analogs of these `death domain` motifs which will provide an even better enhancement of their action, i.e. enhanced cell cytotoxicity, these analogs having one or more amino acids added, deleted or replaced with respect to the naturally occuring sequences.

Detailed Description Text (55):
In a similar fashion it is also possible by the means described herein above to introduce `death domain` motifs or analogs thereof, of the NGF-R or ankyrin 1 into cells in which it is desired to enhance the intracellular effects mediated by NGF-R or ankyrin-1.

Detailed Description Text (56):
Likewise, the present invention also relates to the specific blocking of the effects mediated by the `death domain` motif-containing proteins by blocking the activity of the `death domain` motifs of these proteins, e.g. by the introduction of anti-sense oligonucleotides into cells (as mentioned above) which would block the expression of the `death domain` motifs.

Detailed Description Text (57):
In yet another aspect of the invention there is provided organic compounds, e.g. heterocyclic compounds, which are capable of specifically binding to the `death domain` motifs of one or more `death domain` motif-containing proteins. These organic compounds are well known in the field of pharmaceuticals and are widely used as therapeutic agents which are capable of entering cells (hydrophobicilipophilic compounds) and binding various intracellular proteins or intracellular portions of transmembrane proteins and thereby exerting their effect. These organic compounds may be readily screened and identified by using the `death domain` motifs of the death domain motif-containing proteins, e.g. those of p55 TNF-R, FAS-R. NGF-R, MORT1, ankyrin 1, in standard affinity chromatography procedures or other methods well known in the art.

Detailed Description Text (58):
It should also be mentioned, that the `death domain` motif consists of both general structural features common to all of the various such motifs, i.e. a common scaffold, as well as specific structural features, specific to each of the `death domain` motifs. Accordingly, a preferred drug or pharmaceutically active molecule according to the invention will contain, as active ingredient, naturally occurring proteins or peptides; synthetically produced proteins or peptides including complementary peptides; antibodies; or chemical compounds obtained by screening or design, all of which are characterized by being capable of recognizing the general `death domain` features and one or more of the specific `death domain` features.

Detailed Description Text (59):
The present invention also relates to pharmaceutical compositions comprising recombinant animal virus vectors encoding the `death domain` motif-binding proteins or peptides or the `death domain` motif sequences themselves, which vector also encodes a virus surface protein capable of binding specific target cell (e.g. cancer cells) surface proteins to direct the insertion of the `death domain` motif-binding protein or peptide sequences or the `death domain` motif sequences into the cells. Likewise, the present invention also relates to pharmaceutical compositions comprising organic compounds capable of binding to `death domain` motifs of `death domain` motif-containing proteins.

Detailed Description Text (62):
The `Death Domain` Motif Common to the Receptors p55 TNF-R. FAS-R and NGF-R and to the Proteins MORT1 and Ankyrin 1

Detailed Description Text (63):
Upon the discovery of MORT1 (see co-pending IL 109632, IL 112002 and 112692) it was also discovered that MORT1 contains a region having homology to the previously identified `death domains` of p55 TNF-R and FAS-R (p55DD and FAS-DD, respectively), see IL 109632 and IL 111125). This surprising discovery of a `death domain` motif in a previously unknown protein led to a search for the existence of such a `death domain` motif in other proteins. Surprisingly, such a `death domain` motif was discovered in the low affinity NGF-R and in an apparently unrelated, cytoskeletal protein, ankyrin 1. The `death domain` motifs of all these different proteins share a remarkable homology as is set forth schematically in FIG. 1, which shows a sequence comparison of the `death domain` motifs of the p55 TNF-R, FAS-R, MORT1, low affinity NGF-R and the C terminal part of the regulatory domain in ankyrin 1 (all of human origin). The homology of these `death domain` motifs was defined by the LINEUP and PRETTY programs of the GCG package. Identical and similar residues in three or more of the proteins are boxed. Gaps introduced to maximize alignment are denoted by dots. The significance of this homology was confirmed as follows : (a) Multiple alignment of the `death domain` motif sequences, using the HSSP program of the PredictProtein Service (Sander and Schneider, 1991) showed sequence identities of 21-38% and sequence similarities of 3048%. (b) Searching the Swiss-Prot data bank with a profile created (using the PILEUP, LINEUP and PROFILEMAKE programs of the GCG package) from consensuses of the `death domain` motif sequences in the known p55 R and FAS-R (human, mouse, rat), NGF receptor (human,

rat and chicken) and ankyrins (human and mouse ankyrin 1 and the human ankyrins c and g) sequences and in MORT1 yielded high scores only those sequences that were used for creating the profile (Zscores>8.5 for all of them in search with the "Bioaccelerator" Compugen, Israel).

Detailed Description Text (64):
The above homology search using the PredictProtein Service (PHDsec) and the PRODOM program of the GCG package revealed significant similarity between a region of approximately 65 residues in MORT1. within that part of the molecule that binds to FAS-R, and a region of that same length within the `death domains` of FAS-R and p55-R, (FIG. 1). This part of the `death domain` also shows similarity to a region in the intracellular domain of the low-affinity NGF receptor (Johnson et al., 1986), a receptor whose extracellular domain is known to contain another conserved sequence motif common to FAS-R, the TNF-Rs and other members of the TNF/NGF receptor fanily. It also revealed a previously-unnoticed similarity between this part of the `death domain` and a conserved region in the ankyrins, which are structural proteins that link spectrin-based membrane skeletal proteins to the cytoplasmic domains of integral plasma membrane proteins (Lux et al., 1990; Lambert and Bennett, 1993). That region is located in the N terminal part of the ankyrin regulatory domain, just upstream to that part of the domain whose expression in subject to modulation by alternative splicing, and below the spectrin-binding and membrane binding domains. (The latter domain contains another known sequence motif--the `ankvrin repeat`). The `death domain` motif is distinct from the ankyrin repeat motif that is found in the membrane binding domain of the ankyrins.

Detailed Description Text (65):
The finding of a `death domain` motif in proteins having different intracellular effects suggests that this motif plays a more general role than that implied in the name `death domain` , i.e. this motif occurs in receptors such as p55 TNF-R, FAS-R and the related protein MORT1 which mediate cell cytotoxicity, as well as in the NGF-R which, when inducing death does so only in the absence of ligand (Rabizadeh et al., 1993) and in proteins such as the cytoskeletal ankyrins, not associated with cell cytotoxic effects. One kind of general activity of this `death domain` motif, found so far in three of the proteins containing it, i.e. FAS-R, p55 TNF-R and MORT1 is the ability to self-associate or interact with other proteins that contain this motif.

Detailed Description Text (66):
The discovery of the `death domain` motif in such a wide range of different proteins provides the way for obtaining (as noted herein above and in Example 2 below) proteins or peptides capable of binding to the different (one or more) `death domain` motifs, which proteins and peptides may be used as modulators/mediators of a wide group of regulatory proteins, be they cytokine receptors involved in cell cytotoxic (p55 TNF-R, FAS-R) or growth (NGF-R) effects or related proteins involved in cell cytotoxic effects (MORT1) or regulatory portions of structural proteins involved in the shape/conformational regulation of cells (ankyrins). In a similar fashion, the `death domain` motifs of these various proteins may also be used directly for modulation/mediation of proteins containing such motifs.

Detailed Description Text (68):
Interaction of `Death Domains` of Human p55-TNF-R, FAS-R, TRADD, MORT-1 and RIP

Detailed Description Text (73):
The interactions of the DDs of human p55 TNF-R, FAS-R, TRADD, MORT-1 and RIP were evaluated first by a yeast two-hybrid test. The cDNAs encoding these domains were expressed as fusion proteins with the Gal4 DNA binding and activation domains (DBD and AD constructs) in the yeast SFY526 reporter strain, and the binding of these fusion proteins to each other was assessed by determining .beta.-galactosidase expression by the yeasts. The results of these tests are summarized in FIG. 1 and illustrated diagrammatically in FIG. 3.

Detailed Description Text (74):
The DDs of p55 TNF-R, FAS-R, TRADD and RIP were able to self-associate. The DD of MORT-1 lacked this ability, even though the full length MORT-1 protein does self-associate (Boldin et al., 1995), apparently through an interaction that involves the region upstream of its DD.

Detailed Description Text (75):
The DD of TRADD bound to the DD of p55 TNF-R, but not to the DD of FAS-R, while the DD of MORT-1 behaved in the converse fashion.

Detailed Description Text (76):
The DD of RIP, like the full length RIP protein (Stanger et al., 1995), was able to bind both to the DDs of FAS-R and p55 TNF-R. Binding was significantly weaker, though, than that of the DDs of TRADD and MORT-1 to these receptors. Although RIP wa initially identified by virtue of its binding in a two-hybrid screen to FAS-R (Stanger et al., 1995), this binding is quite weak, and could be observed only when the RIP DD was highly expressed in the yeasts, by introducing it into the AD construct. There was no measurable binding when the DD of RIP was introduced into the DBD construct, which has a lower expression effectivity. A longer RIP insert, corresponding to amino acids 161-372 in the protein, did not bind more effectively to FAS-R

(not shown).

Detailed Description Text (77):
Apart from their observed binding to the DDs of P55 TNF-R or FAS-R, the DDs of each of the three intracellular proteins tested bound also to each other. These interactions were all effective. Notably, the effectivity of binding of the DD of RIP to the DDs of MORT-1 and TRADD was significantly greater than that of its binding to the DDs of p55 TNF-R and FAS-R.

Detailed Description Text (79):
In specificity tests for the two-hybrid assay, we did not observe binding of the DD motifs to any of a number of irrelevant proteins, including SNF 1, the intracellular domain of the human p75 TNF receptor, lamin, cycline D and the DD of the rat low-affinity NGF receptor (not shown). To further assess the binding specificity, we introduced point mutations to the p55 TNF-R, FAS-R, MORT-1 and RIP DDs, at sites corresponding to that of I-225 in the mouse FAS-R sequence. A natrually occurring replacement mutation of this residue, found in lpr.sup.c g mice, abolishes signaling by FAS-R (Itoh and Nagata, 1993; Watanabe-Fukunaga et al., 1992) as well as its interaction with MORT-1 (Boldin et al., 1995; Chinnalyan et al., 1995). Mutation of the corresponding residues in the DDs of human p55 TNF-R (L351N) and FAS-R (V238N) had a similar effect. The mutated proteins were not able to self-associate, nor to bind to TRADD, MORT-1 or RIP. Also, introduction of a replacement mutation to the DD of RIP at the site corresponding to that of the lpr.sup.c g mutation (F308N) resulted in loss of its ability to bind to FAS-R, MORT-1 and TRADD, as well as to self-associate, although the mutated protein bound to the normal RIP DD. On the other hand, in MORT-1 the lpr.sup.c g like mutation (V121N) had only a limited effect. It resulted in less effective binding to FAS-R which, for some reason, was observed only when the mutated protein was introduced into the AD construct but not in the DBD construct.

Detailed Description Text (81):
Although the evidence is still largely indirect, TRADD, MORT-1 and RIP appear to play important roles in the initiation of the cytocidal effect of p55 TNF-R and FAS-R (Cleveland and Ihle, 1995). The binding of these proteins to the receptors, which occurs through their DDs, apparently is required for their contribution to the signaling. A recent study showing that stimulation of FAS-R in cells evokes binding of MORT-1 to this receptor suggests that the DD interactions observed within transfected yeasts also occur within the mammalian cells, and take part in the process of signaling induction (Kischkel et al., 1995). Although the DDs of all the proteins examined have the ability to bind to other DDs, there is clear specificity in this interaction . The DD of TRADD binds to that of p55 TNF-R, but not to the DD of FAS-R. The DD of MORT-1 binds to the DD of FAS-R, but does not bind to the DD of p55 TNF-R. This specificity in the action of proteins that take part in the signaling activity of p55 TNF-R and FAS-R may well contribute to the differences in function of the two receptors.

Detailed Description Text (82):
In addition to their differential binding to the DDs of p55 TNF-R and FAS-R, the DDs of TR ADD and MORT-1 also are able to bind effectively to each other, and both are capable of binding to the DD of REP more effectively than do the DDs of FAS-R or p55 TNF-R. Thus, even though distinct, the signaling cascades affected by TRADD and MORT-1 may well turn to be coordinated through their mutual interactions. The nature of this coordination may vary, depending on the way in which the different interactions of the DD in a given protein affect each other. These interactions may occur together or be exclusive; they may also modulate each other. One possible way for such modulation is indicated by the occurrence in RIP of sequence motifs characteristic of protein kinases. If this protein indeed possesses protein kinase activity, it may turn to be capable of phosphorylating MORT-1 and TRADD upon binding to them, thereby modulating their function. One plausible consequence of the association of TRADD and MORT-1, and of the binding of RIP to both proteins, is integration of their effects, at least in part. This integration may account for the fact that cell death induction by p55 TNF-R and FAS-R exhibit, alongside distinct features, also certain similarities; this could result also in sharing of other activities of the two receptors.

Detailed Description Text (84):
Cloning and Isolation of Proteins which Bind to the `Death Domain` Motifs of `Death Domain` Motif-containing Proteins

Detailed Description Text (85):
To isolate proteins interacting with the `death domain` motifs of `death domain` motif-containing proteins, for example, the `death domain` motifs of p55 TNF-R, FAS-R, NGF-R, MORT1 and ankyrin t, the yeast two-hybrid system (Fields and Song, 1989) may be used as described in co-pending Israel patent application Nos. 109632, 112002 and 112692. Briefly, this two-hybrid system is a yeast-based genetic assay to detect specific protein-protein interactions in vivo by restoration of a eukaryotic transcriptional activator such as GAL4 that has two separate domains, a DNA binding and an activation domain, which domains when expressed and bound together to form a restored GAL4 protein, is capable of binding to an upstream activating sequence which in turn activates a promoter that controls the expression of a reporter gene, such as lacZ or HIS3, the expression of which is readily observed in the cultured cells. In this system the genes for the candidate interacting proteins are cloned into separate expression vectors. In one expression vector the sequence of the one candidate protein is cloned in phase with the sequence of the GAL4 DNA-binding domain to generate a hybrid protein with the GAL4 DNA-binding domain, and in the other vector the sequence of the second candidate protein is cloned in phase with the sequence of the GAL4 activation

domain to generate a hybrid protein with the GAL4-activation domain. The two hybrid vectors are then co-transformed into a yeast host strain having a lacZ or HIS3 reporter gene under the control of upstream GAL4 binding sites. Only those transformed host cells (cotransformants) in which the two hybrid proteins are expressed and are capable of interacting with each other, will be capable of expression of the reporter gene. In the case of the lacz reporter gene, host cells expressing this gene will become blue in color when X-gal is added to the cultures. Hence, blue colonies are indicative of the fact that the two cloned candidate proteins are capable of interacting with each other.

Detailed Description Text (86):
Using this two-hybrid system, the `death domain` motifs may be cloned, separately, into the vector pGBT9 (carrying the GAL4 DNA-binding sequence, provided by CLONTECH, USA, see below), to create fusion proteins with the GAL4 DNA-binding domain. Once the sequence of the `death domain` motif is known, e.g. those shown in FIG. 1, the DNA sequence encoding these motifs may be readily isolated and cloned, by standard procedures into the pGBT9 vector utilizing the vector's multiple cloning site region (MCS).

Detailed Description Text (87):
The above hybrid (chimeric) pGBT9 vectors can then be cotransfected (separately, one cotransfection with each `death domain` motif-containing hybrid together with a cDNA or genomic DNA library from human or other mammalian origin, e.g. a cDNA library from human HeLa cells cloned into the pGAD GH vector, bearing the GAL4 activating domain, into the HF7c yeast host strain (all the above-noted vectors, pGBT9 and pGAD GH carrying the HeLa cell cDNA library, and the yeast strain are purchasable from Clontech Laboratories, Inc., USA, as a part of MATCHMAKER Two-Hybrid System, #PT1265-1). The co-transfected yeasts are then selected for their ability to grow in medium lacking Histidine (His.sup.- medium), growing colonies being indicative of positive transformants. The selected yeast clones were then tested for their ability to express the lacZ gene, i.e. for their LAC Z activity, and this by adding X-gal to the culture medium, which is catabolized to form a blue colored product by .beta.-galactosidase, the enzyme encoded by the lacZ gene. Thus, blue colonies are indicative of an active lacZ gene. For activity of the lacZ gene, it is necessary that the GAL4 transcription activator be present in an active form in the transformed clones, namely that the GAL4 DNA-binding domain encoded by one of the above hybrid vectors be combined properly with the GAL4 activation domain encoded by the other hybrid vector. Such a combination is only possible if the two proteins fused to each of the GAL4 domains are capable of stably interacting (binding) to each other. Thus, the His.sup.+ and blue (LAC Z.sup.+) colonies that are isolated are colonies which have been cotransfected with a vector encoding a `death domain` motif and a vector encoding a protein product of, for example, human HeLa cell origin that is capable of binding stably to a `death domain` motif.

Detailed Description Text (88):
The plasmid DNA from the above His.sup.+, LAC Z.sup.+ yeast colonies can then be isolated and electroporated into E. coli strainHB101 by standard procedures followed by selection of Leu.sup.+ and Ampicillin resistant transformants, these transformants being the ones carrying the hybrid pGAD GH vector which has both the Amp.sup.R and Leu.sup.2 coding sequences. Such transformants therefore are clones carrying the sequences encoding newly identified proteins or peptides capable of binding to the `death domain` motifs. Plasmid DNA was then isolated from these transformed E. coli and retested by:

Detailed Description Text (89):
(a) retransforming them with the original `death domain` motif-containing hybrid plasmids into yeast strain HFU7 as set forth hereinabove. As controls, vectors carrying irrelevant protein encoding sequences, e.g. pACT-lamin or pGBT9 alone can be used for cotransformation with the `death domain` motif-binding protein or peptide encoding plasmids. The cotransformed yeasts can then be tested for growth on His.sup.- medium alone, or with different levels of 3-aminotriazole; and

Detailed Description Text (90):
(b) retransforming the plasmid DNA and original `death domain` motif hybrid plasmids and control plasmids described in (a) into yeast host cells of strain SFY526 and determining the LAC Z.sup.+ activity (effectivity of .beta.-gal formation, i.e. blue color formation). It should be noted that the above noted .beta.-galactosidase (.beta.-gal) expression tests can also be done by a standard filter assay.

Detailed Description Text (92):
Assessment of the Involvement of Sequence Features Characteristic of the `Death Domain` Motif in the Binding of the Cloned Proteins

Detailed Description Text (93):
The cDNA encoding the protein that contains the `death domain` motif will be mutated at the various amino acids that constitute this motif. For example, tryptophan 380 in the intracellular domain of the human low-affinity nerve growth factor receptor (NGF-R) will be replaced with alanine. Such mutation can be performed, for example, by the Kunkel oligonucleotide-directed mutagenesis procedure. The mutated, as well as the wild-type proteins, can be produced in bacteria as fusions with Glutathione S-transferase (GST). The binding of the cloned protein in vitro to the GST fusion with the mutated NGF-R will be compared to its binding to the GST-wild type NGF-R intracellular domain fusion. Abolition of the binding by the mutation will indicate that

the cloned protein indeed recognizes sequence features that are involved in the `death domain` motif. A similar approach will be taken to assess the involvement of the sequence features characteristic of the `death domain` in the function of other reagents that interact with proteins containing this motif, namely antibodies, peptides or organic compounds (See Example 4).

Detailed Description Text (95):
Design of Drugs that Affect `Death Domain` Motif-containing Proteins by Virtue of their Ability to Interact with the `Death Domain`

Detailed Description Text (96):
Organic molecules or peptides that interact with the `death domain` motif of one of the proteins containing this motif will be defined either by screening or by design. Further changes will then be introduced to this molecule to increase the effectivity of its interaction with that specific `death domain` and the ability of the designed compound to affect (enhance or interfere with) the function of the protein containing the `death domain`. Once creating such a molecule and defining the sequence feature of the `death domain` which it recognizes (see Example 3) as well as the conformational features of the `death domain` involved in this recognition (by NMR, X-ray crystallography, etc.), this knowledge can be applied as a starting point for designing drugs that will affect other proteins containing the `death domain` motif. To do so, one should introduce to the designed peptide or organic molecule, besides structural features that allow recognition of those structural features that are common to the `death domain`, also structural features that will dictate specific recognition of the specific `death domain` containing protein.

Detailed Description Text (98):
Analysis of the Biological Activity of the `Death Domain` Motif Binding Proteins, Peptides, Antibodies or Organic Molecules

Detailed Description Text (99):
Once the `death domain` motif binding proteins or peptides have been isolated, e.g. by the procedure of Example 1, they can be tested for their biological activity. In co-pending applications IL 109632, 111125, 112002 and 112692, there is described one such procedure which assays the effect of intracellular domain binding proteins of the cytotoxic effects mediated by the p55 TNF-R, FAS-R and MORT1 (HF1).

Detailed Description Text (100):
Thus, using similar procedures it is possible to determine, firstly, the ability of such `death domain` motif-binding proteins or peptides to associate in vitro with `death domain` motif-containing proteins such as p55 TNF-R, FAS-R, NGF-R, MORT-1, RIP, TRADD and ankyrin 1; and secondly to assess in vivo, using standard cell cytotoxicity assays, whether such `death domain` motif binding proteins or peptides are capable of enhancing or inhibiting the cell cytotoxicity induced by such receptors as p55 TNF-R or FAS-R or proteins such as MORT1.

Detailed Description Text (101):
Likewise, the same tests may also be applied to assay organic compounds (obtained by screening or design, see Example 4); synthetically produced peptides (see Example 4); and antibodies, capable of binding to `death domain` motifs.

Other Reference Publication (19):
Clement, Marie-Veronique et al., "Fas and tumor necrosis factor receptor-mediated cell death: similarities and distinctions." J. Exp. Med., vol. 180, pp. 557-567 (Aug. 1994).

Other Reference Publication (21):
Boldin, Mark P. et al., "A novel protein that interacts with the death domain of fas/apo1 contains a sequence motif related to the death domain.", The Journal of Biological Chemistry, vol. 270, No. 14, pp. 7795-7798 (1995).

Other Reference Publication (22):
Boldin, Mark P. et al., "Self-association of the "Death Domains" of the p55 tumor necrosis factor (tnf) receptor and fas/apo1 prompts signaling for tnf and fas/apo1 effects." The Journal of Biological Chemistry, vol. 270, No. 1, pp. 387-391 (Jan. 1995).

Other Reference Publication (24):
Chinnaiyan, Arul M. et al., "FAAD, a novel death domain-containing protein, interacts with the death domain of fas and initiates apoptosis.", Cell, vol. 81, pp. 505-512 (May 1995).

Other Reference Publication (25):
Stanger, Ben Z. et al., "RIP: a novel protein containing a death domain that interacts with fas/apo1 (cd95) in yeast and causes cell death.", Cell, vol. 81, pp. 513-523 (May 1995).

CLAIMS:

1. An antibody specific to the death domain of a death domain-containing regulatory protein selected from the group consisting of NGF-R, MORT-1 and ankyrin 1.

2. An antibody in accordance with claim 1, wherein said antibody is capable of binding to the death domain of more than one of said death domain-containing regulatory proteins.

3. An antibody in accordance with claim 1 comprising a fragment of an antibody specific to the death domain of one of said death domain-containing regulatory proteins, wherein said fragment is capable of binding said death domain.

4. An antibody in accordance with claim 2 comprising a fragment of an antibody specific to the death domain of one of said death domain-containing regulatory proteins, wherein said fragment is capable of binding the death domain of more than one of said death domain-containing regulatory proteins.

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DOCUMENT-IDENTIFIER: US 6159731 A
TITLE: Daxx, a Fas-binding protein that activates JNK and apoptosis

Brief Summary Text (2):
This invention relates to nucleic acids and encoded polypeptides which bind to Fas and potentiate Fas-mediated apoptosis. The invention also relates to agents which bind the nucleic acids or polypeptides. The invention further relates to methods of using such nucleic acids and polypeptides in the treatment and/or diagnosis of disease.

Brief Summary Text (4):
Fas (also known as CD95 or APO-1) is a widely expressed cell death receptor that has a critical role in the regulation of the immune system and tissue homeostasis. Fas is activated by Fas ligand (FasL), a trimeric transmembrane protein (reviewed by Nagata, Cell 88:355-365, 1997). Fas is thought to have an essential role in deleting autoreactive lymphocytes and maintaining peripheral tolerance. Inherited Fas mutations in humans and mice cause a syndrome of massive lymphoproliferation and autoantibody production (reviewed by Nagata, 1997). Fas-induced apoptosis is also a major mechanism in cytotoxic T lymphocyte-mediated cytolysis and in the maintenance of immune privilege sites (reviewed by Abbas, Cell 84:655-658, 1996). Moreover, depending on the signal from the B cell antigen receptor, Fas may induce either apoptosis or proliferation of B cells in vivo (Rathmell et al., Cell 87:319-329, 1996).

Brief Summary Text (5):
Fas belongs to the tumor necrosis factor (TNF) receptor superfamily, which includes TNF receptor 1 (TNFR1), TNFR2, CD40, and the p75 low affinity NGF receptor; these receptors share characteristic cysteine-rich repeats in their extracellular domains (reviewed by Smith et al., Cell 76:959-962, 1994). The intracellular tails of Fas and TNFR1 share homologous death domains, an approximately eighty amino acid protein motif that is critical for signaling apoptosis (Itoh and Nagata, J. Biol. Chem. 268:10932-10937, 1993; Tartaglia et al., Cell 74:845-853, 1993). Over the last two years, elucidation of the mechanism for Fas-mediated apoptosis has begun (reviewed by Cleveland and Ihle, Cell 81:479-482, 1995; Fraser and Evan, Cell 85:781-784, 1996). FADD, also known as MORT1, is a cytoplasmic protein that has a C-terminal death domain which interacts with Fas and an N-terminal domain that can induce cell death (Chinnaiyan et al., Cell 81:505-512, 1995; Boldin et al., J. Biol. Chem. 270:7795-7798, 1995b). The N-terminus of FADD interacts with MACH/FLICE, an interleukin-1.beta. converting enzyme (ICE) family cysteine protease (caspase) that potently induces apoptosis (Boldin et al., Cell 85:803-815, 1996; Muzio et al., Cell 85:817-827, 1996). Although the details are not yet clear, other caspases, including ICE and CPP32, are sequentially activated to execute the apoptotic dissolution of the cell (Enari et al., Nature 380:723-726, 1996). TNFR1 also interacts with FADD via an adaptor protein termed TRADD (Hsu et al., Cell 84:299-308, 1996). The emerging model from these molecular studies is that Fas, via FADD, directly engages and activates apoptotic ICE family proteases. However, this model fails to explain how Bcl-2 and other physiologic signals may modulate Fas-mediated apoptosis (Fraser and Evan, 1996). It remains possible that other signaling molecules in addition to FADD are involved in Fas-mediated apoptosis.

Brief Summary Text (6):
Fas can also activate the Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) pathway (Latinis and Koretzky, Blood 87:871-875, 1996; Lenczowski et. al., Mol. Cell. Biol. 17:170-181, 1997; Goillot et. al., Proc. Natl. Acad. Sci. USA 94:3302-3307, 1997). Analogous to the MAP kinase cascade, the prototypical JNK/SAPK pathway involves the sequential activation of the proteins MEKK1, SEK1, JNK, and c-Jun. Other targets of the JNK pathway include the transcription factors Elk-1 and ATF-2 (reviewed by Kyriakis and Avruch, Ann. N.Y. Acad. Sci. 766:303-319, 1996). This pathway was initially characterized by the ability of UV irradiation and transforming Ha-Ras to activate the AP-1 transcription factor; subsequently it was shown that TNF-.alpha. and other stress-activated signals may also activate this pathway. The significance of Fas-mediated JNK activation is currently unclear. One hypothesis is that activation of the JNK pathway contributes to Fas-mediated apoptosis (Goillot et. al., 1997). Dominant negative constituents of the JNK pathway can block stress- and TNF-induced apoptosis in several cell lines, suggesting that activation of JNK pathway is required for these apoptotic inducers (Verheij et al., Nature 380:75-79, 1996). Similarly, in PC12 cells that undergo apoptosis in response to nerve growth factor withdrawal, activation of the JNK pathway in concert with the suppression of the ERK pathway is critical to induce programmed cell death (Xia et al.,

Science 270:1326-1331, 1995). Alternatively, Fas-mediated JNK activation may drive cellular proliferation via activation of the proto-oncogene c-Jun and AP-1 transcriptional activity (Rathmell et al., Cell 87:319-329, 1996).

Brief Summary Text (7):
Recently, Liu et. al. have demonstrated that overexpression of FADD, the established downstream signal transducer of Fas, cannot activate JNK but that two other proteins engaged by TNFR1--RIP and TRAF2--are responsible for JNK activation by TNF (Liu et al., Cell 87:565-576, 1996). This raises the question of whether Fas also engages other proteins to activate the JNK pathway.

Brief Summary Text (8):
There exists a need to influence the Fas-mediated apoptosis and JNK signal transduction pathways to treat disease. There also exists a need to identify the gene(s) responsible for increased or decreased signal transduction and to provide therapies for treating diseases resulting from aberrant signal transduction.

Brief Summary Text (10):
We describe herein the molecular cloning and characterization of Daxx, a novel Fas binding protein. Daxx binds to the Fas death domain, yet lacks a death domain of its own. Overexpression of Daxx leads to JNK activation and potentiates Fas-induced apoptosis. The Fas binding domain of Daxx acts as a dominant negative inhibitor of Fas-induced apoptosis and JNK activation. Furthermore, using dominant negative and constitutively active forms of Daxx and FADD, we show that Fas engages two independent pathways to induce cell death: one pathway via Daxx that involves JNK activation and is blocked by Bcl-2, and a second pathway via FADD that is Bcl-2 insensitive.

Brief Summary Text (11):
The invention provides isolated nucleic acid molecules, unique fragments of those molecules, expression vectors containing the foregoing, and host cells transfected with those molecules. The invention also provides isolated polypeptides and agents which bind such polypeptides, including antibodies. The foregoing can be used in the diagnosis or treatment of conditions characterized by the expression of a Daxx nucleic acid or polypeptide. The invention also provides methods for identifying pharmacological agents useful in the diagnosis or treatment of such conditions. Here, we present the cDNA cloning of a 81.4 kDa Fas-associated protein, Daxx, a protein which potentiates Fas-mediated apoptosis.

Brief Summary Text (12):
According to one aspect of the invention, an isolated nucleic acid molecule is provided. The molecule hybridizes under stringent conditions to a molecule consisting of the nucleic acid sequence of SEQ ID NO:1. The isolated nucleic acid molecule codes for a polypeptide which binds to Fas. The invention further embraces nucleic acid molecules that differ from the foregoing isolated nucleic acid molecules in codon sequence to the degeneracy of the genetic code. The invention also embraces complements of the foregoing nucleic acids. In preferred embodiments, the isolated nucleic acid molecule comprises nucleotides 1-2358 of SEQ ID NO:1 or nucleotides 1-2340 of SEQ ID NO:4. In particularly preferred embodiments, the isolated nucleic acid molecule comprises nucleotides 25-2241 of SEQ ID NO:1 or nucleotides 1-2220 of SEQ ID NO:4.

Brief Summary Text (18):
According to another aspect of the invention, there are provided isolated polypeptides which selectively bind a complex of a first polypeptide, preferably a Daxx polypeptide or fragment thereof, bound to Fas. In certain embodiments the first polypeptide comprises the sequence of SEQ ID NO:2 or SEQ ID NO:5, and more preferably consists essentially of the sequence of SEQ ID NO:2 or SEQ ID NO:5. In preferred embodiments, the isolated binding polypeptides include antibodies and fragments of antibodies (e.g., Fab, F(ab).sub.2, Fd and antibody fragments which include a CDR3 region which binds selectively to the complex of the first polypeptide and Fas). According to still another aspect of the invention, methods for increasing or decreasing Fas-mediated apoptosis in a mammalian cell are provided. The methods involve administering to a mammalian cell an amount of Daxx-encoding nucleic acid or polypeptide, or an inhibitor of Daxx activity, effective to increase or decrease, respectively, Fas-mediated apoptosis in the mammalian cell. In certain embodiments, the inhibitor is a dominant negative fragment of Daxx polypeptide, especially the 112 C-terminal amino acids of SEQ ID NOs:2 or 5, or an antisense nucleic acid which inhibits the expression of Daxx.

Brief Summary Text (21):
The invention in a further aspect involves methods for increasing or decreasing Daxx-Fas or Daxx-ASK1 binding activity in a subject. An agent that selectively binds to an isolated nucleic acid molecule of the invention or an expression product thereof is administered to a subject in need of such treatment, in an amount effective to decrease Daxx-Fas or Daxx-ASK1 binding activity in the subject. Preferred agents are antisense nucleic acids, including modified nucleic acids, and polypeptides, such as a dominant negative variant of Daxx. For increasing Daxx-Fas or Daxx-ASK1 binding activity in a subject, an isolated nucleic acid molecule of the invention or an expression product thereof is administered to a subject in need of such treatment, in an amount effective to increase Daxx-Fas or Daxx-ASK1 binding activity in the subject.

Brief Summary Text (22):
According to another aspect of the invention, methods for decreasing Fas-mediated apoptosis in a cell are provided. The methods comprise contacting the cell with an amount of an agent which decreases the Fas-mediated apoptosis potentiating activity of Daxx effective to decrease Fas-mediated apoptosis in the cell. In certain embodiments, the agent is a vector which comprises a promoter active in the cell operably linked to a nucleic acid molecule selected from the group consisting of a nucleic acid molecule which encodes a Daxx dominant negative polypeptide, and a nucleic acid which encodes a Daxx antisense nucleic acid molecule. In other embodiments, the agent is a Daxx antisense oligonucleotide. In still other embodiments, the agent is a compound which inhibits the binding of Daxx to a polypeptide selected from the group consisting of Fas and ASK1.

Brief Summary Text (23):
According to still another aspect of the invention, methods for increasing apoptosis in a cell are provided. The methods comprise contacting the cell with an amount of an agent which increases apoptosis in the cell, wherein the agent is selected from the group consisting of a vector which expresses Daxx, or a fragment thereof which has Fas-mediated apoptosis-inducing activity, a Daxx polypeptide, or a fragment thereof which has Fas-mediated apoptosis-inducing activity, a vector which expresses a fragment of Daxx polypeptide which has constitutive apoptosis activity, and a fragment of Daxx polypeptide which has constitutive apoptosis activity. In embociments wherein the agent is a vector which expresses a fragment of Daxx polypeptide which has constitutive apoptosis activity or a fragment of Daxx polypeptide which has constitutive apoptosis activity, the fragment of Daxx polypeptide which has constitutive apoptosis activity preferably is selected from group consisting of DaxxDC, DaxxC501, and Daxx 501-625.

Brief Summary Text (24):
The invention in another aspect provides methods for treating a condition characterized by abnormal Fas-mediated apoptosis. Such methods comprise administering to a subject having the condition an amount of an agent which decreases the Fas apoptosis potentiating activity of Daxx effective to reduce Fas-mediated apoptosis in the subject. Preferably the agent is a vector which comprises a promoter active in the tissue affected by the condition operably linked to a nucleic acid molecule selected from the group consisting of a nucleic acid molecule which encodes a Daxx dominant negative polypeptide, and a nucleic acid which encodes a Daxx antisense nucleic acid molecule. In other preferred embodiments the agent is a Daxx antisense oligonucleotide or a compound which inhibits the binding of Daxx to a polypeptide selected from the group consisting of Fas and ASK1. In certain embodiments of the foregoing methods, the condition is selected from the group consisting of 1) allograft tissue rejection, 2) graft-versus-host disease, 3) fulminant hepatitis, and 4) cancers which increase the amount of Fas apoptosis, including NK lymphoma and large granular lymphocytic leukemia. In other embodiments of the foregoing methods, the condition is apoptosis of T cells (e.g. by immune privileged cells such as tumor cells) and the agent is delivered to T cells.

Brief Summary Text (25):
In yet another aspect of the invention, methods for treating a condition characterized by insufficient apoptosis are provided. The methods comprise administering to a subject having the condition an amount of an agent which increases Daxx apoptosis activity effective to increase apoptosis in the subject. In certain embodiments, the condition is characterized by insufficient Fas-mediated apoptosis, and the agent increases Fas-mediated apoptosis activity of Daxx effective to increase Fas-mediated apoptosis in the subject; preferred embodiments include those wherein the condition is selected from the group consisting of septic shock/sepsis, autoimmune disease including autoimmune lymphoproliferative syndrome, liver hyperplasia and abnormal lymphoproliferation including lymphoma. In other preferred embodiments, the agent is selected from the group consisting of a Daxx polypeptide and a vector having a promoter operably linked to Daxx nucleic acid.

Brief Summary Text (27):
According to another aspect of the invention, methods are provided for identifying lead compounds for a pharmacological agent useful in the diagnosis or treatment of disease associated with Daxx Fas binding activity. The methods involve forming a mixture of a Daxx polypeptide or fragment thereof containing a Fas death domain binding site, a protein which interacts with the foregoing Fas death domain binding site, and a candidate pharmacological agent. The mixture is incubated under conditions which, in the absence of the candidate pharmacological agent, permit a first amount of selective binding of the protein which interacts with the Daxx Fas death domain binding site by the Daxx polypeptide containing a Fas death domain binding site. A test amount of selective binding of the protein which interacts with the Daxx Fas death domain binding site by the Daxx polypeptide containing a Fas death domain binding site then is detected. Detection of an increase in the test amount of selective binding of the Fas death domain by the Daxx polypeptide containing a Fas death domain binding site in the presence of the candidate pharmacological agent relative to the first amount indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which increases selective binding of the Fas death domain by the Daxx polypeptide containing a Fas death domain binding site. Detection of a decrease in the foregoing activity in the presence of the candidate pharmacological agent indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which decreases selective binding of the Fas death domain by the Daxx polypeptide containing a Fas death domain binding site. Preferably the protein containing a Fas death domain binding site is a Daxx polypeptide which comprises the amino acid sequence of the 112 C-terminal amino acids of SEQ ID NO:2 or of SEQ ID NO:5. Similar methods are provided for identifying lead compounds for a pharmacological agent which reduces or enhances Daxx protein binding to ASK1.

Brief Summary Text (28):
According to another aspect of the invention, a method for diagnosing a disorder characterized by aberrant Fas apoptosis or JNK signal transduction is provided. The method includes contacting a biological sample isolated from a subject suspected of having the disorder with a first agent that binds to a first protein selected from the group consisting of Daxx, Fas and ASK1. The method also includes the step of isolating a protein complex bound to the first agent, such as by immunoprecipitation, electrophoresis, chromatography and the like. The method also includes determining the proteins in the protein complex as a determination of the disorder. Where the complex does not include Daxx and Fas or ASK1, the subject can be diagnosed as having the disorder. Preferably the step of determining the proteins in the protein complex includes contacting the protein complex with a second agent that binds to a second protein selected from the group consisting of Daxx, Fas and ASK1, wherein the second protein is not the first protein. In certain embodiments of the foregoing methods, the first agent and/or the second agent is an antibody.

Brief Summary Text (30):
Thus an object of the invention is to provide compounds that desirably influence the Fas-mediated apoptosis and JNK signal transduction pathways.

Brief Summary Text (32):
Still another object of the invention is to provide diagnostics and research tools relating to Daxx, Fas and JNK.

Drawing Description Text (2):
FIG. 1 shows the interaction of clone A and Daxx with Fas death domain in yeast. FIG. 1A is a schematic representation of the cytoplasmic domain of murine Fas. FIG. 1B shows interaction in the two-hybrid system. FIG. 1C shows that the C-terminus of clone A interacts with Fas death domain.

Drawing Description Text (4):
FIG. 3 depicts the interaction of Daxx with Fas in vitro and in mammalian cells. FIG. 3A shows binding of in vitro translated .sup.35 S-Daxx to GST-fusion proteins. FIG. 3B depicts binding of full length and truncated .sup.35 S-Daxx to GST and GST-FasDD. FIG. 3C depicts the association of HA-Daxx and HA-DaxxDC with the GST fusion of Fas intracellular tail (GST-FasIC) in 293 cells.

Drawing Description Text (5):
FIG. 4 shows that Daxx potentiates Fas-induced apoptosis. FIG. 4A shows the morphology of normal and apoptotic 293 and HeLa cells. FIG. 4B demonstrates that Daxx potentiates Fas-induced apoptosis in 293 cells. FIG. 4C shows that Daxx potentiates Fas-induced apoptosis in HeLa cells. FIG. 4D demonstrates that L929 cells stably overexpressing Daxx have accelerated specific apoptosis in response to Fas.

Drawing Description Text (7):
FIG. 6 relates deletion analysis of Daxx. FIG. 6A shows apoptosis and JNK activation by Daxx deletion mutants. FIG. 6B shows that DaxxC inhibits Fas-induced apoptosis. FIG. 6C shows that DaxxC inhibits Fas-induced JNK activation.

Drawing Description Text (8):
FIG. 7 shows that Daxx and FADD activate distinct apoptotic pathways. FIG. 7A shows the inhibition of Fas-induced apoptosis by DaxxC and FADD(80-205). FIG. 7B shows that FADD(80-205) fails to inhibit Fas-induced JNK activation. FIG. 7C shows that DaxxC does not inhibit FADD-mediated apoptosis.

Drawing Description Text (9):
FIG. 8 depicts the inhibition profile of Daxx- and FADD-induced apoptosis. FIG. 8A shows the inhibition profile of Fas-induced apoptosis in L929, 293, and HeLa cells. FIG. 8B shows the inhibition of profile of Fas+Daxx, Daxx501-625 and FADD in 293 cells. FIG. 8C depicts two pathways of Fas signaling that induce cell death.

Drawing Description Text (10):
FIG. 9 shows that a Fas mutant from autoimmune lymphoproliferative syndrome has a selective defect in Daxx binding. FIG. 9A shows the binding of Fas wildtype and mutant proteins to Daxx and FADD. FIG. 9B shows the equal expression of the proteins in the experimental system.

Drawing Description Text (11):
FIG. 10 shows that Daxx interacts with ASK1 and recruits ASK1 to Fas. FIG. 10A shows that Daxx binds ASK1 in mammalian cells. FIG. 10B shows that endogenous ASK1 is recruited to Fas in a ligand-dependent manner.

Detailed Description Text (8):
The present invention in one aspect involves the cloning of a cDNA encoding a Daxx Fas binding protein. The sequence of the

mouse gene is presented as SEQ ID NO:1, and the predicted amino acid sequence of this gene's protein product is presented as SEQ ID NO:2. The sequence of the human gene is presented as SEQ ID NO:4, and the predicted amino acid sequence of this gene's protein product is presented as SEQ ID NO:5. Analysis of the sequence by comparison to nucleic acid and protein databases determined that Daxx is a completely novel protein with no significant sequence similarity to death domains or other protein motifs.

Detailed Description Text (16):
The invention also provides isolated unique fragments of SEQ ID NO:1 or SEQ ID NO:4 or complements of thereof. A unique fragment is one that is a `signature` for the larger nucleic acid. It, for example, is long enough to assure that its precise sequence is not found in molecules outside of the Daxx nucleic acids defined above. Unique fragments can be used as probes in Southern blot assays to identify such nucleic acids, or can be used in amplification assays such as those employing PCR. As known to those skilled in the art, large probes such as 200 nucleotides or more are preferred for certain uses such as Southern blots, while smaller fragments will be preferred for uses such as PCR. Unique fragments also can be used to produce fusion proteins for generating antibodies or determining binding of the polypeptide fragments, as demonstrated in the Examples, or for generating immunoassay components. Likewise, unique fragments can be employed to produce nonfused fragments of the Daxx polypeptides, useful, for example, in immunoassays or as a competitive binding partner of the Fas and/or other polypeptides which bind to the Daxx polypeptides, for example, in therapeutic applications. Unique fragments further can be used as antisense molecules to inhibit the expression of Daxx nucleic acids and polypeptides, particularly for therapeutic purposes as described in greater detail below.

Detailed Description Text (18):
As mentioned above, the invention embraces antisense oligonucleotides that selectively bind to a nucleic acid molecule encoding a Daxx polypeptide, to decrease Fas binding by Daxx. This is desirable in virtually any medical condition wherein a reduction in Fas binding activity of Daxx is desirable, including to reduce apoptosis. Antisense Daxx molecules, in this manner, can be used to slow down or arrest the proliferation of cancer cells in vivo.

Detailed Description Text (31):
The invention also permits the construction of Daxx gene "knock-outs" in cells and in animals, providing materials for studying certain aspects of Fas binding activity and signal transduction.

Detailed Description Text (35):
The invention embraces variants of the Daxx polypeptides described above. As used herein, a "variant" of a Daxx polypeptide is a polypeptide which contains one or more modifications to the primary amino acid sequence of a Daxx polypeptide. Modifications which create a Daxx variant can be made to a Daxx polypeptide 1) to reduce or eliminate an activity of a Daxx polypeptide, such as Fas binding or stimulation of JNK activity; 2) to enhance a property of a Daxx polypeptide, such as protein stability in an expression system or the stability of protein-protein binding; or 3) to provide a novel activity or property to a Daxx polypeptide, such as addition of an anti genic epitope as provided in the Examples, or addition of a detectable moiety. Modifications to a Daxx polypeptide are typically made to the nucleic acid which encodes the Daxx polypeptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids or non-amino acid moieties. Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety, such as biotin, addition of a fatty acid, and the like. Modifications also embrace fusion proteins comprising all or part of the Daxx amino acid sequence.

Detailed Description Text (39):
The skilled artisan will realize that conservative amino acid substitutions may be made in Daxx polypeptides to provide functionally equivalent variants of the foregoing polypeptides, i.e, the variants retain the functional capabilities of the Daxx polypeptides. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution which does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g. Molecular Cloning: A Laboratory Manual, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, or Current Protocols in Molecular Biology, F. M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Exemplary functionally equivalent variants of the Daxx polypeptides include conservative amino acid substitutions of SEQ ID NO:2 and/or SEQ ID NO:5, particularly conservative substitutions of amino acids other than the 101 or 112 C-terminal amino acids of SEQ ID NO:2 and/or SEQ ID NO:5, which include sequences which are sufficient for Daxx binding to Fas. However, conservative substitutions of those amino acids can be made as well. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

Detailed Description Text (42):
The end result of the expression of a dominant negative polypeptide in a cell is a reduction in function of active proteins. One of ordinary skill in the art can assess the potential for a dominant negative variant of a protein, and using standard mutagenesis

techniques to create one or more dominant negative variant polypeptides. For example, given the teachings contained herein of a Daxx polypeptide, one of ordinary skill in the art can modify the sequence of the Daxx polypeptide by site-specific mutagenesis, scanning mutagenesis, partial gene deletion or truncation, and the like. See, e.g., U.S. Pat. No. 5,580,723 and Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989. The skilled artisan then can test the population of mutagenized polypeptides for diminution in a selected activity (e.g., activation of apoptosis or the JNK pathway) and for retention of a desired activity (e.g., Fas binding activity of Daxx). Other similar methods for creating and testing dominant negative variants of a protein will be apparent to one of ordinary skill in the art.

Detailed Description Text (43):
Dominant negative Daxx proteins include variants in which a portion of the amino terminus of the Daxx protein has been mutated or deleted to reduce or eliminate downstream effects of Daxx-Fas binding, i.e. enhanced Fas-mediated apoptosis. For example, the C-terminal portion of Daxx (e.g., the 112 C-terminal amino acids of SEQ ID NO:2) can bind to Fas, and acts as a dominant negative inhibitor of Fas-induced apoptosis and JNK activation (see Examples below).

Detailed Description Text (46):
The invention also makes it possible isolate proteins having particular Daxx binding domains, such as Fas and TNFRI, by the binding of such proteins to Fas death domain binding sequences present in the Daxx polypeptide, as disclosed herein. The identification of the Fas binding site of Daxx also permits one of skill in the art to block the binding of a protein having a death domain, such as Fas, with a binding partner having a Fas binding site, such as Daxx or a fragment of Daxx which retains the Fas binding site. For example, binding of the proteins can be effected by introducing into a biological system in which the proteins bind (e.g., a cell) a polypeptide including a Fas binding site in an amount sufficient to block the binding of Daxx to Fas. The identification of the Fas binding site in Daxx also enables one of skill in the art to prepare modified proteins, using standard recombinant DNA techniques, which can bind to proteins containing a Fas death domain. For example, when one desires to target a certain protein to the inner membrane surface where proteins containing a death domain, such as Fas, are localized, one can prepare a fusion polypeptide of the certain protein and the Daxx Fas binding site. Other proteins selectively bind to additional binding sites located in the Daxx polypeptide. These include proteins which activate the JNK pathway.

Detailed Description Text (48):
The invention, therefore, embraces peptide binding agents which, for example, can be antibodies or fragments of antibodies having the ability to selectively bind to Daxx polypeptides alone or in a complex with a binding partner such as Fas. Antibodies include polyclonal and monoclonal antibodies, prepared according to conventional methodology. Where antibodies which bind a complex of a Daxx polypeptide and a binding partner, such as Fas, are desired, the complex is used as the immunogen for preparation of the antibody. The complex can be cross-linked according to standard protein cross-linking techniques to provide a stable molecule as the immunogen. Thus an antibody which bind the complex are preferably those antibodies which recognize epitopes on both the Daxx polypeptide and binding partner components of the complex. Alternatively, complex-specific antibodies can be those antibodies which recognize an epitope on one of the complex components which is specific to the conformation of the component in the complex. For example, Daxx may assume a preferred conformation when bound to Fas which creates one or more antigenic epitopes not present in the unbound Daxx polypeptide.

Detailed Description Text (54):
Phage display can be particularly effective in identifying binding peptides useful according to the invention. Briefly, one prepares a phage library (using e.g. m13, fd, or lambda phage), displaying inserts from 4 to about 80 amino acid residues using conventional procedures. The inserts may represent, for example, a completely degenerate or biased array. One then can select phage-bearing inserts which bind to the Daxx polypeptide or a complex of Daxx and a binding partner such as Fas. This process can be repeated through several cycles of reselection of phage that bind to the Daxx polypeptide or complex. Repeated rounds lead to enrichment of phage bearing particular sequences. DNA sequence analysis can be conducted to identify the sequences of the expressed polypeptides. The minimal linear portion of the sequence that binds to the Daxx polypeptide or complex can be determined. One can repeat the procedure using a biased library containing inserts containing part or all of the minimal linear portion plus one or more additional degenerate residues upstream or downstream thereof. Yeast two-hybrid screening methods also may be used to identify polypeptides that bind to the Daxx polypeptides. Thus, the Daxx polypeptides of the invention, or a fragment thereof, or complexes of Daxx and a binding partner can be used to screen peptide libraries, including phage display libraries, to identify and select peptide binding partners of the Daxx polypeptides of the invention. Such molecules can be used, as described, for screening assays, for purification protocols, for interfering directly with the functioning of Daxx and for other purposes that will be apparent to those of ordinary skill in the art.

Detailed Description Text (55):
A Daxx polypeptide, or a fragment which contains Fas binding site, also can be used to isolate their native binding partners, including, e.g., the Fas protein that complexes with Daxx. Isolation of binding partners may be according to well-known methods. For example, isolated Daxx polypeptides can be attached to a substrate, and then a solution suspected of containing Fas protein may be applied to the substrate. If the binding partner for Daxx polypeptides is present in the solution, then it will bind to the substrate-bound Daxx polypeptide. The binding partner then may be isolated. Other proteins which are binding partners for

Daxx, such as other proteins which contain Fas death domains may be isolated by similar methods without undue experimentation.

Detailed Description Text (57):
The invention further provides methods for reducing or increasing Fas-mediated apoptosis in a cell. Such methods are useful in vitro for altering Fas-mediated apoptosis, for example, in testing compounds for potential to block aberrant Fas-mediated apoptosis. In preferred embodiments, the methods involve contacting the cell with an agent which modulates the Fas-mediated apoptosis potentiating activity of Daxx in an amount effective to modulate such apoptosis, i.e., to increase or decrease Fas-mediated apoptosis. As used herein, "Fas-mediated apoptosis potentiating activity of Daxx" or "Daxx Fas-mediated apoptosis potentiating activity" means the effect which Daxx has on the apoptotic processes induced by Fas. As demonstrated herein, Daxx potentiates Fas-mediated apoptosis (and Fas-mediated JNK activation). Increasing Fas-mediated apoptosis in a cell by, e.g., introducing a Daxx polypeptide in the cell, or decreasing Fas-mediated apoptosis in a cell by, e.g., introducing a dominant negative Daxx polypeptide or Daxx antisense nucleic acid in the cell, can be used to provide a model system for testing compounds which affect Fas-mediated apoptosis. Such methods also are useful in the treatment of conditions which result from excessive or deficient Fas-mediated apoptosis. As known in the art, and as shown in the Examples, Fas-mediated apoptosis can be measured readily by determining membrane blebbing, pyknosis, cell body condensation, and nuclear fragmentation as judged by Hoechst staining. Other methods will also be known to one of skill in the art.

Detailed Description Text (58):
The agent for modulation of Fas-mediated apoptosis will be different for increasing or decreasing Fas-mediated apoptosis. For example, in methods for decreasing Fas-mediated apoptosis, agents include compounds which reduce the effect of Daxx, including Daxx dominant negative polypeptides, such as fragments of Daxx which bind to Fas but do not transduce the apoptotic signal, and anti-Daxx antibodies. Agents also include compounds which reduce the expression of Daxx, such as antisense Daxx nucleic acids including oligonucleotides. The skilled artisan will understand that identification of such agents can proceed via standard methods of the art. For example, to determine the effect of antisense Daxx nucleic acid molecules, one can contact a Daxx-expressing cell with such molecules and detemine the expression of Daxx before and after the contacting. A reduction in Daxx nucleic acid or polypeptide expression indicates that the antisense molecule effectively reduced the expression of Daxx and thus would be a candidate for an agent for decreasing Fas-mediated apoptosis. Identification of Daxx fragments which modulate Fas-mediated apoptosis can include assays such as determining the binding of Daxx to Fas in the presence and the absence of such Daxx fragments. Daxx fragments which reduce the binding of Daxx and Fas are candidates for agents which reduce the effect of Daxx on Fas-mediated apoptosis. Other similar methods standard to the molecular biology and drug discovery arts can also be employed to identify agents for modulating the effects of Daxx on Fas-mediated apoptosis. Thus, the "agent" is not limited to nucleic acids and polypeptides, but also embraces small molecules which could interfere with, or enhance, the effects of Daxx on Fas-mediated apoptosis.

Detailed Description Text (59):
Methods for increasing apoptosis in a cell are also provided. In these methods, a cell is contacted with an agent which increases apoptosis in the cell. For these methods, an agents include Daxx polypeptides which potentiate Fas-mediated apoptosis and nucleic acids encoding such polypeptides, and also Daxx polypeptides which have consitutive apoptosis-inducing activity (e.g., which do not require Fas/Fas-L binding for apoptosis) and nucleic acids encoding such polypeptides. In particular, the latter Daxx molecules, those which have constitutive apoptotic activity, include DaxxDC, DaxxC501 and Daxx501-625. Methods for identifying other such agents are provided herein, such as the methods described for identification of Fas-mediated apoptosis modulating agents above, and in the Examples below. One of ordinary skill in the art will understand that standard methods of molecular biology which are used for determining the fragments of polypeptides and nucleic acids that have a certain activity can be used to further dissect Daxx for fragments which have constitutive apoptosis activity. Such methods include mutagenesis, including site-directed mutagenesis, preparation of fragments by restriction endonuclease or exonuclease digestion, and the like, followed by expression of the Daxx fragment and testing for constitutive apoptosis activity as described above for Fas-mediated apoptosis.

Detailed Description Text (60):
The foregoing agents which modulate the effect of Daxx on Fas-mediated apoptosis can also be used in the treatment of conditions characterized by abnormal Fas-mediated apoptosis. The methods involve administering to a subject having such a condition an amount of an agent which decreases the Fas-mediated apoptosis potentiating activity of Daxx effective to reduce the Fas-mediated apoptosis.

Detailed Description Text (61):
Several conditions have been identified which result from abnormal Fas-mediated apoptosis. These conditions include: allograft tissue rejection in which the allograft is destroyed by apoptosis (Smyth et al., Transplantation 62:1529-1532, 1996), graft-versus-host disease (Via et al., J. Immunol. 157:5387-5393, 1996; Baker et al., Proc. Acad. Nat'l. Sci. USA 94:1366-1371, 1997), fulminant hepatitis (Ogasawara et al., Nature, 364:806-809, 1993; Tanaka et al., J. Immunol. 158:2303-2309, 1997), and certain cancers, including NK lymphoma and large granular lymphocytic leukemia (Tanaka et al., Nature Med. 2:317-322,

1996). Other conditions which result from Fas-mediated apoptosis can be identified by the methods employed to identify the foregoing conditions. In general, such methods can include assays of apoptosis in cells derived from such subjects, in the presence and in the absence of compounds which block Fas activity. Other assays will be known to those skilled in the art. For conditions in which reduced Fas-mediated apoptosis results in a disease state, such as septic shock/sepsis, autoimmune disease resulting from a lack of elimination of B cells which produce autoantibodies (Elkon et al, Curr. Opin. Immunol. 8:852-859, 1996), liver hyperplasia (Adachi et al., Nat. Genet. 11:294-300, 1995), and abnormal lymphoproliferation (see Nagata, 1997 and references therein), agents which increase Fas-mediated apoptosis, or which have constitutive apoptotic activity, can be administered. Such agents are described in greater detail above and in the Examples below.

Detailed Description Text (62):
The subject can be monitored before, during and/or after administering an agent to modulate the effect of Daxx on Fas-mediated apoptosis. Various parameters can be monitored, such as the modulation of Fas-mediated apoptosis, the reduced or increased synthesis and/or steady state levels of Daxx, and the relevant clinical parameters of the particular condition. Methods for measuring apoptosis have been extensively described in the literature, both for in vitro model systems and in connection with conditions resulting from aberrant Fas-mediated apoptosis. Methods for measuring Daxx nucleic acids and/or polypeptides are set forth herein.

Detailed Description Text (65):
The result of the detection step (the first result) is then compared with a control result to provide a diagnostic measure of the disorder. For comparisons in which the first result is greater than the control result, the diagnosis would indicate increased expression of Daxx. For comparisons in which the first result is lesser than the control result, the diagnosis would indicate decreased expression of Daxx. Additional diagnostic methods relate to determining the proteins present in Daxx protein complexes. As shown herein, Daxx binds to Fas and to ASK1. In certain conditions, the Daxx pathway is disrupted at the Fas-Daxx or Daxx-ASK1 binding step. Thus, for example, determining Daxx-Fas or Daxx-ASK1 binding can be predictive of such disorders. It is also possible to diagnose subsets of such disorders by examining the binding of FADD to Fas. As shown herein, certain Fas mutants bind to FADD but not Daxx, while other Fas mutants fail to bind to FADD and Daxx. The former mutants are found in autoimmune patients who also have a high correlation of lymphomas. Thus, by examining these protein interactions, one can diagnose the disorder and also predict the outcome of the disorder, thereby permitting treatment which is better suited to a particular subject.

Detailed Description Text (72):
The invention further provides efficient methods of screening compounds for agents active at the level of a cellular function modulated by Daxx or a Daxx fragment. In particular, such functions include Fas-mediated apoptosis, JNK signal transduction, and formation of a Daxx-Fas protein complex or a Daxx-ASK1 protein complex. Generally, the screening methods involve assaying for compounds which interfere with a Daxx activity such as Daxx-Fas or Daxx-ASK1 binding, etc. Such methods are adaptable to automated, high throughput screening of compounds. The target indications for agents detected by the screening methods are limited only in that the target cellular function be subject to modulation by alteration of the formation of a complex comprising a Daxx polypeptide or fragment thereof and one or more natural Daxx intracellular binding targets, such as Fas or other protein including a Fas death domain. Target indications include cellular processes modulated by JNK, Fas, and other Fas death domain-containing proteins described above.

Detailed Description Text (73):
A wide variety of assays for compounds are provided, including, labeled in vitro protein-protein binding assays, immunoassays, cell-based assays such as two- or three-hybrid screens, expression assays, etc. For example, three-hybrid screens are used to rapidly examine the effect of transfected nucleic acids on the intracellular binding of Daxx or Daxx fragments to specific binding partners such as Fas or ASK1. The transfected nucleic acids can encode, for example, combinatorial peptide libraries or antisense molecules. Convenient reagents for such assays, e.g., GAL4 fusion proteins, are known in the art. An exemplary cell-based assay involves transfecting a cell with a nucleic acid encoding a Fas-binding Daxx polypeptide fused to a GAL4 DNA binding domain and a nucleic acid encoding a Fas protein including a death domain fused to a transcription activation domain such as VP16. The cell also contains a reporter gene operably linked to a gene expression regulatory region, such as one or more GAL4 binding sites. Activation of reporter gene transcription occurs when the Daxx and Fas fusion polypeptides bind such that the GAL4 DNA binding domain and the VP16 transcriptional activation domain are brought into proximity to enable transcription of the reporter gene. Compounds which modulate a Daxx-Fas binding are then detected through a change in the expression of reporter gene. Methods for determining changes in the expression of a reporter gene are known in the art. Similar assays for Daxx-ASK1 binding also are provided.

Detailed Description Text (74):
Daxx and/or fragments used in the methods, when not produced by a transfected nucleic acid are added to an assay mixture as an isolated polypeptide. Daxx polypeptides preferably are produced recombinantly, although such polypeptides may be isolated from biological extracts. Recombinantly produced Daxx polypeptides include chimeric proteins comprising a fusion of a Daxx protein with another polypeptide, e.g., a polypeptide capable of providing or enhancing protein-protein binding, sequence

specific nucleic acid binding (such as GAL4), enhancing stability of the Daxx polypeptide under assay conditions, or providing a detectable moiety, such as green fluorescent protein. A polypeptide fused to a Daxx polypeptide or fragment may also provide means of readily detecting the fusion protein, e.g., by immunological recognition or by fluorescent labeling.

Detailed Description Text (75):
The assay mixture is comprised of a natural intracellular Daxx binding target (e.g. a Daxx binding partner such as a Fas protein or ASK1). While natural Daxx binding targets may be used, it is frequently preferred to use portions (e.g., peptides or nucleic acid fragments) or analogs of the Daxx binding target (i.e., agents which mimic the Daxx binding properties of the natural binding target for purposes of the assay) so long as the portion or analog provides binding affinity and avidity to the Daxx fragment measurable in the assay.

Detailed Description Text (84):
The invention provides Daxx-specific binding agents, methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, Daxx-specific agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving Daxx, e.g., JNK activation, Fas-Daxx complex formation, etc. Novel Daxx-specific binding agents include Daxx-specific antibodies and other natural intracellular binding agents identified with assays such as two hybrid screens, and non-natural intracellular binding agents identified in screens of chemical libraries and the like.

Detailed Description Text (90):
Anti-murine Fas Jo2 antibody was the generous gift of S. Nagata (Ogasawara et al., Nature 364:806-809, 1993). Murine TNF-.alpha. (Genzyme, Cambridge, Mass.), monoclonal antibody M2 against Flag epitope (Kodak/IBI), and anti-JNK-1 antibody C17 (Santa Cruz Biotech, Santa Cruz, Calif.) were obtained from the indicated sources. HeLa, 293, and L929 were obtained originally from American Type Culture Collection (ATCC, Rockville, Md.). To establish the L/Daxx cell line, HA-Daxx vector was cotransfected with pBabe-puro into L929 cells using lipofectamine-mediated gene transfer (GIBCO-BRL, Gaithersburg, Md.). Resistant cells were selected in media containing 2.5 .mu.g/ml of puromycin. Positive clones were identified by immunoblot analysis using anti-HA antibody 12CA9. The L929 cells expressing mFas (L/Fas) were established by transfecting pRc/CMV-mFas into L929 cells and subsequently selecting for resistant cells in 600 .mu.g/ml G418. Resistant clones were then screened for Fas expression by FACScan using Jo2 antibody.

Detailed Description Text (92):
DNA fragments for most plasmid constructs were obtained by PCR amplification using Pfu polymerase (Stratagene, La Jolla, Calif.) and primers incorporated with appropriate restriction sites and epitope tags as needed. The fragments for LexA and transcription activator fusions were cloned into plasmid pEG202 and pJG4-5 (Gyuris et al., Cell 75:791-803, 1993), respectively. The I225N mutation in LexA-lpr.sup.cg was made by site-directed mutagenesis using a two-step PCR protocol (Higuchi et al., Nucleic Acids Res. 16:7351-7367, 1988). GST constructs were made in pGEX vector (Pharmacia, Piscataway, N.J.). In vitro translation constructs were made in pET3a (Novagen, Madison, Wis.) or pBluescript (Stratagene). Daxx(-162) was obtained by digestion of a full length Daxx construct with HindIII. For expressing proteins in mammalian cells, full length Daxx and FADD, and mutants thereof, were cloned into pEBB, a derivative of pEF-BOS (Mizushima and Nagata, Nucleic Acids Res. 18:5322, 1990), with a hemagglutinin (HA) epitope-tag at the 5' end. Full length murine Fas was cloned into pEBB and pRc/CMV (Invitrogen, Carlsbad, Calif.). Fas intracellular region (amino acids 165 to 306) was fused to GST in pEBG, a derivative of pEBB expressing GST. Full length murine TNFR1 was cloned in pEBB. Each construct was confirmed by restriction digestion, partial DNA sequence, and for plasmids expressing proteins in yeast and mammalian cells, by immunoblot analysis.

Detailed Description Text (105):
For L929 apoptosis assay in FIG. 8, 2.5.times.10.sup.5 cells/well of L/Fas cells were seeded in 6-well plates. On the next day cells were co-transfected with 200 ng of pHook-1 plasmid (Invitrogen) and 400 ng of crmA, Bcl-xL or SEK (AL) with lipofectamine. The pHook-1 construct expresses a single chain antibody (sFv) against the hapten phOx, which allows selection of transfected cells by using phOx-conjugated magnetic beads. Twenty four hours after transfection cells were removed from the dish in 1 ml of PBS/3 mM EDTA. Magnetic beads (1.5.times.10.sup.6) were added to the cells and incubated at 37.degree. C. for 30 min. The cells were then washed 3 times in media, counted and plated in duplicate in 96 well plates (.about.5000 cells/well). After culture for 14-16 hours, Fas-killing was induced using Jo2 antibody (1 .mu.g/ml) and 0.5 .mu.g/ml of actinomycin D and measured by counting the number of surviving cells in four random fields 24 hours later. Efficiency of transfection and cell selection were monitored by cotransfection of pCMV-lacZ.

Detailed Description Text (107):
HeLa and 293 cells were transfected in 60 mm dishes with Flag-JNK plus the indicated expression plasmids by the calcium phosphate method. Approximately 24 hours after transfection cells were serum starved for 14-16 hours. Cells transfected with Fas were treated with 0.5 mg/ml of Jo2 antibody for 30 min. To test for the effect of protease inhibitors, cells were treated with 0.5 mg/ml of Jo2 and 100 mM of ICE inhibitor Z-Val-Ala-Asp-CH2F or CPP32 inhibitor Z-Asp-Glu-Val-Asp-CH2F (Enzyme Systems Products, Calif.) for 30 min. JNK-1 was immunoprecipitated with anti-Flag antibody, and in vitro kinase assay with 1

.mu.g of GST-cJun (1-79) was performed as previously described (Khosravi-Far et al., Mol. Cell. Biol. 16:3923-3933, 1996). The kinase reaction was then stopped with 2.times.SDS polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Proteins were separated on 15% SDS-PAGE in duplicate gels. One set was stained to check for equivalent GST-cJun loading. This gel was then dried and exposed to film for 4-6 hours. Another gel was immunoblotted with anti-FLAG antibody to visualize expression of JNK-1.

Detailed Description Text (108):
JNK1 kinase activity in L929 cells stably expressing Fas or Daxx was measured by immunoprecipitation of the endogenous JNK-1 using anti-JNK-1 C-17 antibody. TNFR1- and Fas-transfected cells were treated with 20 ng/ml TNF-.alpha. for 10 minutes and 0.5 .mu.g/ml Jo2 for 30 minutes, respectively. The kinase assay was then carried out as described above.

Detailed Description Text (111):
Two-hybrid Screen for Novel Fas Interacting Proteins

Detailed Description Text (112):
To identify novel Fas interacting proteins, we performed a two-hybrid screen with the death domain of murine Fas fused to the DNA binding protein LexA (LexA-mFasDD). A plasmid library of fusions between a transcription activator domain and cDNAs from human HeLa cells was screened for interaction with LexA-mFasDD in a yeast reporter strain. LexA constructs containing the indicated amino acids (in parenthesis) of receptors expressed similar level of fusion proteins in yeast. Colony color and .beta.-galactosidase units were determined as described in the Experimental Procedures. One group of positive interactors, typified by clone A21, interacted strongly with FasDD. However, it interacted poorly with either Fas-lp.sup.cg, an Ile224Asn mutation in the Fas death domain which abrogates Fas signaling and causes lymphoproliferation in mice (reviewed by Nagata, 1997) or with Fas-FD8, a functionally inactive deletion mutation of the Fas death domain (Itoh and Nagata, 1993) (FIG. 1B). Sequence analysis of clone A21 revealed it to encode a portion of a novel protein. The activation hybrid Act-DaxxC501 contains amino acids 501 to 739 of Daxx.

Detailed Description Text (113):
In the two-hybrid system, clone A21 also interacted with the intracellular domain of human Fas and the death domain of TNFR1, but not with the intracellular region of CD40, a closely related receptor that lacks a death domain (FIG. 1B). The sequence C-terminal to the Fas death domain has been shown to inhibit the cytotoxicity of Fas death domain (Itoh and Nagata, 1993) and it also inhibits the binding of FADD to Fas (Chinnaiyan et al., 1995). The presence of this inhibitory region had no effect on the binding of clone A21 to Fas because clone A21 interacted equally well with FasDD and FasIC (FIG. 1B).

Detailed Description Text (114):
We mapped the Fas interaction domain on Clone A21 to the C-terminal 112 amino acids by deletion analysis (FIG. 1C). Amino acids contained in each activation hybrids are indicated. This region showed no evident sequence similarity to death domains, suggesting that the interaction between clone A21 and Fas is not through a homotypic death domain association.

Detailed Description Text (117):
Using clone A21 as the probe, we cloned a cross-hybridizing full length murine cDNA (SEQ ID NO:1). Sequence analysis revealed an open reading frame able to encode a protein of 739 amino acids (SEQ ID NO:2) with a predicted molecular mass of 81.4 kDa (FIG. 2A). The C-terminus of this protein is homologous to clone A21. The open reading frame of murine Daxx follows an in-frame stop codon and begins with a Kozak consensus sequence. The regions enriched for acidic residues and proline are underlined. The partial human cDNA sequence from A21 (SEQ ID NO: 3) is shown below the mouse sequence. Identical amino acids between mouse and human sequences are indicated by "-". The full length human Daxx cDNA was also cloned and sequenced (SEQ ID NO: 5). We call this gene Daxx for Fas death domain associated protein. A database search using BLAST revealed that Daxx is a novel protein with no significant sequence similarity to any other protein. Daxx contains a region of 62 amino acids with a high content (71%) of glutamic acid and aspartic acid and contains two small proline-rich regions (FIG. 2A).

Detailed Description Text (118):
To determine the tissue distribution of Daxx, we performed a Northern analysis with a Daxx C-terminal probe. A mouse multiple tissue Northern blot was probed with a C-terminal 0.7 kb fragment of Daxx and a human .beta.-actin cDNA. A 2.6 kb transcript, consistent with the length of the open reading frame, was detected in various adult mouse tissues (FIG. 2B). The expression of Daxx appeared uniform, with the exception of stronger expression in testis. Shorter hybridizing transcripts were also detected in liver, kidney, and testis. Cell lines from many tissues have been reported to support the ability of ectopically expressed Fas to induce apoptosis, suggesting that the downstream signaling mechanism is present in most tissues. Similarly, FADD is expressed ubiquitously in adult tissues (Boldin et al., 1995b; Chinnaiyan et al., 1995).

Detailed Description Text (120):
Daxx Interacts with Fas Both in vitro and in vivo

Detailed Description Text (121):
In the two-hybrid system, the Daxx C-terminal region interacted strongly with Fas, confirming that Daxx is the functional homolog of clone A21 (FIG. 1B). We then tested the binding of full length murine Daxx protein in vitro and in mammalian cells. In vitro translated, .sup.35 S-labeled Daxx bound to immobilized glutathione S-transferase (GST) fusion proteins of Fas death domain and TNFR1 intracellular tail but not to immobilized GST or GST-CD40 intracellular tail or GST-Fas lpr.sup.cg death domain (FIG. 3A). Positions of MW standards (in kDa) are shown at left. Coomassie stained GST fusion proteins from the same gel were aligned to show protein levels. Daxx migrated with an apparent molecular weight of approximately 120 kDa on SDS-PAGE; this slower than expected migration may reflect the high content of acidic residues in Daxx. In the GST pull-down assay, .sup.35 S-Daxx bound to GST-FasDD but only very weakly to GST-TNFR1. This discrepancy with the two-hybrid result (FIG. 1) may be due to the nonlinear readout of the two-hybrid system. Deletion of 162 amino acids from the C-terminus of Daxx abrogated binding to GST-FasDD while the C-terminal 112 amino acids (DaxxC) was sufficient to bind GST-FasDD (FIG. 3B), consistent with the two-hybrid results. Daxx(-162) was missing C-terminal 162 aa of Daxx; DaxxC corresponded to aa 628-739. Input of .sup.35 S-Daxx and .sup.35 S-Daxx(-162) proteins in binding assays are shown in lane 1 and 2, respectively. GST fusion proteins are shown on the bottom panel.

Detailed Description Text (122):
To determine whether Daxx interacted with Fas in mammalian cells, human embryonic kidney 293 cells were cotransfected with constructs expressing hemagglutinin-tagged Daxx (HA-Daxx) and GST-Fas intracellular tail (GST-FasIC). The presence of HA-Daxx and HA-DaxxDC in extracts was verified by immunoblotting for HA (bottom panel, FIG. 3C). HA-Daxx was coprecipitated with GST-FasIC but not with GST by glutathione beads. Again, this interaction was dependent on the C-terminus of Daxx (FIG. 3C). GST-FasIC was also able to coprecipitate HA-tagged Fas death domain, confirming that death domains may multimerize. Because Fas is overexpressed and thereby activated, we are uncertain whether Daxx binds to the inactive Fas. Collectively, these data show that the C-terminus of Daxx mediates an interaction between the Fas death domain and Daxx, and that this interaction is likely to occur directly and in vivo.

Detailed Description Text (124):
Daxx Potentiates Fas-mediated Apoptosis

Detailed Description Text (125):
To study the role of Daxx in Fas signaling, we chose 293 cells and HeLa cells. Both are sensitive to Fas- and TNF-mediated apoptosis, and their normally flat morphology facilitates the scoring of apoptotic cells, characterized by membrane blebbing, pyknosis, and cell body condensation (FIG. 4A). Cells scored to be apoptotic by morphology also exhibited nuclear condensation and fragmentation as judged by Hoechst staining. HeLa and 293 cells were transiently transfected with various expression constructs (as indicated) and an expression construct for .beta.-galactosidase; at defined times after the transfection, cells were stained for .beta.-galactosidase activity to mark the transfected cells and scored for apoptotic morphology. In 293 cells, transient overexpression of Fas induced apoptosis in a dose-dependent and saturable manner (FIG. 4B). Indicated amount (in .mu.g) of pEBB-Fas and pEBB-HA-Daxx plasmids were cotransfected with 0.5 .mu.g of pCMV-lacZ. The total amount of transfected DNA was made constant by adding vector DNA. The cells were stained with X-gal 20 hours after transfection and analyzed for apoptotic morphology as described in the Experimental Procedures above. Fas activation in the absence of activating ligand is due to a documented propensity of death domains to multimerize (Boldin et al., J. Biol. Chem. 270:387-391, 1995a). However, the addition of activating anti-Fas antibodies, Jo2, did not increase cell death in Fas-transfected 293 cells, implying that a function downstream of receptor activation may be limiting. Overexpression of Daxx by itself did not induce apoptosis, but Daxx coexpression significantly enhanced Fas-mediated apoptosis (FIG. 4B). Parallel experiments with TNFR1 did not show any enhancement of apoptosis by Daxx, consistent with the much lower affinity of Daxx for TNFR1 (FIG. 3A).

Detailed Description Text (126):
In HeLa cells, transient transfection of Fas led to robust, dose-dependent and saturable cell death (FIG. 4A, C), which was further enhanced by the addition of Jo2. Transfection and specific apoptosis were done and measured as in 293 cells except that X-gal staining was done at 24 hrs after transfection. As in 293 cells, overexpression of Daxx alone did not induce apoptosis in HeLa cells. In the range where apoptosis is proportional to input Fas DNA, coexpression of Daxx significantly increased Fas-mediated apoptosis (FIG. 4C), suggesting that Daxx activity may be a rate limiting step downstream of receptor engagement.

Detailed Description Text (127):
In an analogous approach to assess the function of Daxx, we established murine fibroblast L929 cell lines that stably overexpress Daxx (L/Daxx). L/Daxx cells are substantially more susceptible to Fas killing compared to vector transfected cells (L/EBB). L/EBB and L/Daxx were transfected with 1 .mu.g of pEBB-Fas, pEBB-TNFR1, or pRK-FADD plus 0.2 .mu.g of pCMV-lacZ. Specific apoptosis was determined as in FIG. 4B at indicated time after transfection. Similar results were obtained with multiple L/Daxx lines. This stimulation effect appeared to be a kinetic one: the L/Daxx culture had greater than three-fold more apoptotic cells 24 hours after Fas transfection compared to L/EBB, but L/EBB cells caught up by 48 hours after transfection (FIG. 4D).

TNF-.alpha.-, TNFR1-, or FADD-mediated apoptosis was not increased in L/Daxx cells (FIG. 4D and not shown). Therefore, L/Daxx cells are not generally more sensitive to apoptosis, but are specifically sensitized to the Fas signal, suggesting that Daxx is a mediator of Fas-induced killing.

Detailed Description Text (130):
The lack of death domain homology or constitutive cell death activity suggests that Daxx may play a different role from previously identified death domain binding proteins in Fas signaling. Fas has been reported to activate the JNK pathway (Latinis and Koretzky, 1996), which is required in certain cell lines for the analogous TNF-.alpha.-induced apoptosis (Verheij et al., 1996). We therefore analyzed the ability of Fas and Daxx to activate the kinase activity of JNK and JNK-dependent transcription. In transient transfection assays in 293 cells, Fas activated JNK-1, the major JNK activity in cells (Derijard et al., Cell 76:1025-1037, 1994). Flag-tagged JNK1 (Flag-JNK) and the indicated plasmids (1 .mu.g each) were cotransfected into 293 cells. Fas-induced JNK activation was not blocked by the serpin ICE inhibitor crmA (FIG. SA), a peptide ICE inhibitor Z-VAD, or a peptide CPP32 inhibitor Z-DEVD. FADD overexpression did not induce JNK (FIG. 5A). Therefore Fas activation of JNK is not secondary to FADD activity or apoptosis. Interestingly, Daxx overexpression activated JNK-1 to a level similar to that of Fas (FIG. 5A). To assay endogenous JNK activation by Fas and Dax, we used L929 cells stably expressing murine Fas (L/Fas) and the L/Daxx and L/EBB cells. In L/Fas cells, Fas-induced JNK activation was observed approximately 15 minutes after Fas ligation and reached maximal activity in about one hour (FIG. 5A). L/Daxx cells had constitutive activation of JNK activity compared to L/EBB cells (FIG. 5B), and the level of JNK activation correlated with the level of Daxx overexpression in various L/Daxx cell lines.

Detailed Description Text (131):
As an independent measure of JNK activity, we tested the ability of Fas and Daxx to stimulate signaling to SRF (Serum Response Factor). JNK can phosphorylate and activate SRF independent of the MEK/MAPK pathway, and the level of JNK activation in vivo can be assayed using a reporter gene driven by SRE-L, a derivative of SRE that specifically binds SRF (Hill et. al., 1995). In 293 cells, Fas induced SRF-dependent transcription about 4-fold, and Daxx induced it about 6-fold. TNF-.alpha., a known inducer of the JNK pathway, stimulated the SRF-reporter gene to a level similar to that induced by Fas or Daxx (FIG. 5C).

Detailed Description Text (135):
To further dissect Daxx signaling, we asked which regions of Daxx were required for its three activities: Fas binding, enhancement of apoptosis, and activation of JNK. We have already determined that the C-terminal 112 amino acids of Daxx (DaxxC) are necessary and sufficient for Fas binding (FIG. 3B). Significantly, Daxx mutants missing either the C-terminus (DaxxDC) or N-terminus 500 amino acids (DaxxC501) acquired a modest constitutive cell death activity for 293 cells in the absence of Fas (FIG. 6A). Apoptosis assay: 3 .mu.g of each Daxx mutant construct was transfected into 293 cells as above. HeLa cells and L929 cells were not sensitive to this activity. The result in 293 cells suggested that deletion of either end of Daxx activated a normally latent cell death activity. Because DaxxDC is unable to bind Fas death domain (FIGS. 3B and 3C), this cell death activity is likely to be independent of Fas or other death domain proteins. Further deletions revealed that amino acids 501 to 625, which lie immediately N-terminal to the Fas binding domain, contained most of the cell death activity (FIG. 6A).

Detailed Description Text (138):
DaxxC Is a Dominant Negative Inhibitor of Fas-mediated Apoptosis and JNK Activation

Detailed Description Text (139):
Deletion mutagenesis showed that DaxxC, the C-terminal 112 amino acids of Daxx, was necessary and sufficient to bind Fas but more N-terminal domains were required to activate JNK and cell death. Thus, we tested whether DaxxC can act as a dominant negative inhibitor of endogenous Daxx by competing with its binding to Fas. We chose to use HeLa cells in these experiments because the cells have a robust response to transfected Fas (FIG. 4C) and are the source of clone A21 from the two-hybrid screen. HeLa cells were transfected with 0.5 .mu.g pEBB-Fas and pCMV-lacZ and the indicated amount (in .mu.g) of HA-Daxx and HA-DaxxC. Total amount of transfected DNA was made constant by adding pEBB. Jo2 (12.5 ng/ml) was added 16 hrs later. X-gal staining was done at 24 hrs after transfection. In FIG. 6B, we show that expression of DaxxC gave a dose-dependent suppression of Fas-mediated apoptosis. Fas-induced c-Jun phosphorylation was also inhibited by DaxxC in HeLa cells (transient transfection of 1 .mu.g of each indicated plasmid with 1 .mu.g of Flag-JNK and in vitro JNK assay was done as above) and in 293 cells (FIG. 6C). To address the specificity of DaxxC, we then coexpressed full-length Daxx with DaxxC and asked if this combination now reversed the dominant negative effect. If DaxxC were binding other death domain containing proteins (e.g. FADD), coexpression of full length Daxx would further titrate FADD away from Fas and inhibit apoptosis. Instead, coexpression of Daxx with Fas and DaxxC gave a dose-dependent rescue of Fas-induced apoptosis (FIG. 6B). This result argues that the only functions made deficient by DaxxC are those of intact Daxx, implying that DaxxC specifically competes with endogenous Daxx but not other proteins for binding to Fas. These results suggest that endogenous Daxx is required for Fas-induced apoptosis and JNK activation.

Detailed Description Text (141):

Daxx and FADD Define Two Distinct Fas-Mediated Signaling Pathways

Detailed Description Text (142):
Because Daxx and FADD are both required for Fas-induced apoptosis, we assessed how these two effectors may be related to each other by a dominant negative approach. The FADD death domain, FADD(80-205), has been shown to block Fas-induced death presumably by preventing the binding of endogenous FADD (Chinnaiyan et al., 1996). HeLa cells were transfected with 0.5 .mu.g of pEBB-Fas, pCMV-lacZ, and plasmids (in .mu.g) expressing the indicated proteins. Jo2 (12.5 ng/ml) was added 16 hrs later; X-gal staining was done 24 hrs after transfection. JNK kinase activity was assayed after transient transfection of 2 .mu.g of each of the indicated plasmids with 2 .mu.g of Flag-JNK. We found that FADD(80-205) partially inhibited Fas-induced death (FIG. 7A, lanes 1-3) but did not inhibit JNK activation (FIG. 7B). Moreover, the effect of FADD(80-205) on cell death was not reversed by coexpression of excess Daxx (FIG. 7A, lane 4). These results contrast with the effects of DaxxC (FIG. 6) and suggest that Daxx and FADD bind independently to Fas and activate distinct pathways. Consistent with this interpretation, FADD-induced cell death is not blocked by DaxxC (FIG. 7C). HeLa cells were transfected with the indicated amount (in .mu.g) of FADD and DaxxC and assayed as FIG. 4C. HeLa cells were transfected with the indicated amount (in .mu.g) of FADD and DaxxC and assayed as described above. In addition, DaxxC plus FADD(80-205) inhibited Fas-induced cell death substantially more than saturating amounts of either dominant negative protein alone (FIG. 7A, lane 7). Thus, Daxx and FADD activate apoptosis downstream of Fas by distinct but cooperative pathways.

Detailed Description Text (143):
Fas-mediated apoptosis can be inhibited by crmA and, in some cell types, by Bcl-2, a negative regulator of cell death (Enari et al., Nature 375:78-81, 1995; Los et al., Nature 375:81-83, 1995; Tewari and Dixit, J. Biol. Chem. 270:3255-3260, 1995; Itoh et al., J. Immunol. 151:621-627, 1993; Lacronique et. al., Nature Med. 2:80-86, 1996). To dissect the apoptotic pathways initiated by overexpression of Fas, Daxx, and FADD, the ability of crmA, Bcl-2, SEK(AL) and TAM67 to block each apoptotic inducer was tested. SEK(AL) and TAM67 are dominant negative inhibitors of the JNK pathway. SEK1 is the kinase that phosphorylates and activates JNK; SEK(AL) encodes a mutant that has a single mutation at the ATP-binding site, abrogating the kinase activity (Sanchez et al., Nature 372:794-798, 1994). TAM67 is a variant of c-Jun in which amino acids 3-122 have been deleted. This mutant can dimerize and bind DNA but lacks a transcriptional activation domain (Brown et al., Oncogene 9791-799, 1994). First, the ability of the panel of inhibitor genes to block Fas was tested in several cell types commonly used in apoptosis studies. Transfection and apoptosis analysis in L/Fas cells were performed as described above. 293 cells were cotransfected with 2 .mu.g pEBB-Fas plus vector or plasmids expressing indicated genes (2 .mu.g each) and pCMV-lacZ (0.5 .mu.g) as above. HeLa cells were transfected with 1 .mu.g pEBB-Fas plus plasmids expressing the indicated genes (3 .mu.g each) and pCMV-lacZ (0.5 .mu.g); Jo2 (12.5 ng/ml) addition and X-gal staining was done as above. It was found that Fas-induced apoptosis in L929, 293, and HeLa cells can be blocked by crmA and Bcl-2-type inhibitors, but only 293 cells and L929 cells required the JNK pathway for Fas-induced apoptosis (FIG. 8A). This result is consistent with the work of Liu et. al., who reported that the JNK pathway appeared dispensable for TNF-.alpha.-induced apoptosis in HeLa cells (Liu et al., Cell 87:565-576, 1996). Kolesnick and colleagues have previously shown that TNF-.alpha.-induced apoptosis is inhibited in U937 human monoblastic leukemia cells that stably express TAM67 (Verheij et al., 1996); assayed as above, we found that these cells are also resistant to Fas-mediated apoptosis. Taken together, these observations indicate that the requirement for the JNK pathway in Fas-mediated apoptosis is cell-type specific. It should be noted that this type of dominant negative experiment gives a positive result only if the protein in question is uniquely required for a particular process. In a case where a negative result (no inhibition) is obtained, it may be that the protein in question is involved but is functionally redundant or in great excess. The continued discovery of JNK relatives makes such scenarios plausible (Gupta et al., EMBO J. 15:2760-2770, 1996).

Detailed Description Text (144):
Next, the same panel of inhibitor genes was tested on FADD and Daxx-induced apoptosis. Since full length Daxx does not induce apoptosis by itself, two alternative strategies were used: the apoptotic response of Fas plus Daxx in 293 cells (where a large fraction of the apoptotic response is Daxx-dependent, FIG. 4B) and the apoptotic response of Daxx 501-625, the smallest domain that has constitutive cell death activity in 293 cells (FIG. 6A), were examined. 293 cells were transiently transfected with Fas, Daxx, Daxx 501-625, or FADD (1 .mu.g each) plus empty vector or plasmids expressing the indicated apoptotic inhibitor genes (3 .mu.g each) and pCMV-lacZ (0.5 .mu.g) as FIG. 4B. Daxx-dependent apoptosis was blocked by crmA, Bcl-2, and required the JNK pathway, which paralleled the inhibition profile of Fas in 293 cells (FIG. 8B). In contrast, FADD, which can not activate JNK, was inhibited only by crmA, but not by Bcl-2, SEK(AL), or TAM67. Similarly, Hsu et al. have shown that TRADD-mediated apoptosis is not blocked by Bcl-2 (Hsu et al. Cell 81:495-504, 1995). Consistent with the two pathway model, the residual Fas-induced death remaining after either DaxxC or FADD(80-205) treatment are qualitatively different: the apoptosis remaining after FADD(80-205) treatment is Bcl-2 sensitive but the apoptosis remaining after DaxxC treatment is not (FIG. 7A, lanes 8 and 9). These results suggest that Fas activates two distinct cell death pathways--one via FADD that is Bcl-2 insensitive and a second one via Daxx that activates JNK and is Bcl-2 sensitive.

Detailed Description Text (147):
ALPS is a disease characterized by early onset lymphadenopathy, lymphoproliferation, and autoantibody production leading to hemolytic anemia, thrombocytopenia and other autoimmune symptoms. Most patients harbor defects in the Fas apoptosis

pathway (Fisher et al., Cell 81:935-946, 1995). As shown above, Daxx is a downstream signaling protein in the Fas pathway.

Detailed Description Text (148):
The role of Daxx in ALPS was evaluated by screening Fas mutations derived from ALPS patients for Daxx binding. Expression constructs for epitope-tagged wildtype and mutant (mu1 and mu2) human Fas cDNAs (Fisher et al., 1995) were cotransfected with pCI-FLAG-FADD or pRK5-FLAG-hDAXX and pRK5-crmA (Hsu et al., 1996) in 293T cells as described above. CrmA prevents the induction of apoptosis and allows the accumulation of transfected proteins. Twenty-four hours after transfection cells were lysed in IP-lysis buffer (Hsu et al., 1996). Immunoprecipitation with anti-AU1 antibodies (BAbCO, Berkeley, Calif.) and immunoblotting with anti 1A shows the results of the immunoblotting and FIG. 1B shows that Daxx, FADD and Fas mutants were equally expressed in all cell lysates.

Detailed Description Text (149):
FIG. 1A shows that the mu1 and mu2 Fas mutants have different binding properties. While the mu2 Fas mutation disrupts binding to both Daxx and FADD, the mu1 Fas mutation disrupts only Fas-DAXX binding. Therefore a selective defect in Daxx binding by Fas (such as in mu1) is sufficient to cause ALPS. The selective Daxx defect conferred by Fas mu1 us correlated with a distinct clinical disease. In the analysis of ALPS mu1 pateints, the mu1 mutation cosegregated with a high incidence of lymphomas in addition to autoimmune disease in the affected families; lymphomas are not generally observed with ALPS patients. Therefore determining the Daxx binding property of Fas mutants in ALPS has prognostic value. Dysregulation of the Daxx pathway also may be involved in other autoimmune diseases or tumorigenesis.

Detailed Description Text (152):
As demonstrated above, Daxx activates the Jun N-terminal kinase (JNK) and enhances Fas-induced apoptosis. It has now been discovered that apoptosis signal-regulating kinase 1 (ASK1; Ichijo et al., Science 275:90-94, 1997), an upstream kinase in the JNK pathway, is a downstream target of Daxx.

Detailed Description Text (154):
To determined if endogenous ASK1 is recruited to Fas, 1.5.times.10.sup.7 L929/Fas cells were incubated in the presence or absence of 2 .mu.g/ml anti-Fas antibody Jo2 (Pharmingen,) for 30 minutes. Cells were washed once in ice-cold PBS and lysed in 1 ml IP-lysis buffer. Post-nuclear supernatant was immunoprecipitated with 40 .mu.l of protein A/G-agarose (Santa Cruz) for 3 hours at 4.degree. C. In samples without Jo2 preincubation, 2 .mu.g/ml isotype-matched control antibody (FIG. 10B, lane 1) of Jo2 (FIG. 10B, lane 2) were added after cell lysis. Immunoprecipitates were washed 5 times with 500 .mu.l lysis buffer, resolved by 7.5% SDS-PAGE and immunoblotted for ASK1 by an ASK1-specific antiserum. As shown in FIG. 10, lane 3, ASK1 is recruited to Fas in a ligand-dependent manner, presumably through the Fas-Daxx and Daxx-ASK1 interactions.

Detailed Description Text (155):
The interaction of ASK1 with Daxx can be used to evaluate mutations in either gene in human diseases such as autoimmune disorders. The Daxx-ASK1 complex also can be used to screen for compounds which reduce the binding of these two proteins and thereby reduce activity of the Daxx pathway. The demonstration that endogenous ASK1 can be recruited to Fas provides an assay for testing Fas and Daxx function in patients having autoimmune disease to determine if the Fas-Daxx pathway is involved.

CLAIMS:

1. An isolated nucleic acid molecule

(a) which hybridizes under stringent conditions to a molecule consisting of the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:4 and which codes for a polypeptide which binds to Fas,

(b) nucleic acid molecules that differ from the nucleic acid molecules of (a) in codon sequence due to the degeneracy of the genetic code, and

(c) complements of (a) and (b).

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L3: Entry 3 of 3 File: USPT May 12, 1998

DOCUMENT-IDENTIFIER: US 5750653 A
TITLE: Protein, FAF1, which potentiates Fas-mediated apoptosis and uses thereof

Abstract Text (1):
The present invention identifies a novel, Fas-associated factor 1 termed FAF1 which potentiates Fas-induced cell killing. The invention provides FAF1 nucleic acid and polypeptide compositions as well as methods of using these compositions in the therapeutic treatment of diseases resulting from dysregulation in apoptosis. Also provided are cells carrying and expressing the nucleic acid compositions and methods of using these cells to screen for agonists and antagonists of Fas-mediated apoptosis. Methods of isolating FAF1-interacting proteins are disclosed.

Brief Summary Text (4):
The protein, Fas, mediates apoptosis. A cell surface receptor, Fas plays an important role in the development and function of the immune system. Malfunction of the Fas system causes lymphoproliferative disorders and accelerates autoimmune diseases. Exacerbation of Fas-mediated apoptosis may cause tissue destruction.

Brief Summary Text (6):
The present invention provides the identification and isolation of a novel Fas-associated factor 1, termed FAF1, and the FAF1-encoding DNA. As a cytoplasmic protein, FAF1 was shown herein to bind to the wild type but not the inactive point mutant of Fas. FAF1 specifically interacts with the cytoplasmic domain of wild type Fas and potentiates Fas-induced cell killing. FAF1 is a signal transducing molecule in the regulation of apoptosis.

Brief Summary Text (7):
The FAF1 nucleic acids and polypeptides find many uses. It would be desirable to be able to control apoptosis by enhancing or decreasing the susceptibility of individual cell types to undergo apoptosis, especially when dysregulation of the process leads to disease. In particular, it would be desirable to provide therapeutic intervention in disease conditions where apoptosis is dysregulated due to Fas or FAF1 malfunction. Blockage or activation of FAF1 function in apoptosis can be used for example, in the treatment of cancer, immune disorders such as autoimmune diseases, infectious diseases, and myocardial infarction and neuronal infarction in cardiovascular diseases. Heretofore, such therapeutic approaches involving FAF1 were not possible. The present discovery of FAF1 fulfills these and other needs.

Brief Summary Text (9):
One aspect of this invention is to provide an isolated nucleic acid comprising at least 85% sequence identity with the nucleotide sequence of SEQ ID NO:1, an allelic or species variation thereof, or a fragment thereof. A nucleic acid comprising the nucleotide sequence of SEQ ID NO:1 is provided. Also provided is a recombinant DNA molecule comprising the nucleotide sequence of SEQ ID NO:1 or a fragment thereof. In one embodiment, the recombinant DNA molecule encodes a FAF1-GAL4 transactivation domain fusion protein. A cell is provided which contains the recombinant DNA molecule comprising the nucleotide sequence of SEQ ID NO:1 or a fragment thereof.

Brief Summary Text (10):
Another aspect of the invention is to provide an isolated polypeptide comprising at least 85% sequence identity with the sequence of SEQ ID NO:2, an allelic or species variation thereof, or a fragment thereof. The polypeptide can be provided in a kit. The polypeptide may further comprise an influenza virus HA epitope tag. An isolated polypeptide comprising the sequence of SEQ ID NO:2 is also provided. In a specific embodiment, the polypeptide is one capable of associating with the cytoplasmic domain of Fas.

Brief Summary Text (11):
Yet another aspect of the invention is to provide an isolated polypeptide comprising the sequence of SEQ ID NO:2, an allelic or species variation thereof, or a fragment thereof, wherein the isolated polypeptide is a fusion protein. A FAF1-GAL4 activation

domain fusion protein is specifically provided. In one aspect, the fusion protein comprises a tag, or a product of a second gene or fragment of that second gene product. A fusion protein is provided wherein the tag is GST, an epitope tag or an enzyme or wherein the second gene is lacZ.

Brief Summary Text (14):
Another aspect of the invention is to provide a method of modulating or blocking Fas activity comprising providing a Fas-interacting domain polypeptide of FAF1 in a cell expressing Fas protein wherein said Fas-interacting domain polypeptide of FAF1 binds to said Fas protein to block Fas activity. In one embodiment, the Fas-interacting domain polypeptide of FAF1 is provided by introducing an expression vector encoding a Fas-interacting domain polypeptide of FAF1 into the Fas expressing cell. A method of activating FAF1-mediated apoptosis in a cell is disclosed, the method comprising providing a constitutively active FAF1 to the cell.

Brief Summary Text (15):
An important aspect of the invention is a method of screening for an agonist or an antagonist of FAF1, comprising contacting a cell expressing both Fas and FAF1 with a test molecule, activating Fas, and analyzing the cell for any effects on apoptosis, increased apoptosis indicative that the test molecule is an agonist and decreased or loss of apoptosis indicative that the test molecule is an antagonist. The test molecules can be peptides, oligonucleotides, lipids, toxins, hormones, small proteins, drugs and compounds from plant or animal sources and recombinantly produced substances. In a specific embodiment, peptide libraries are screened.

Brief Summary Text (16):
The cells are analyzed for effects on apoptosis such as cell membrane blebbing and/or DNA fragmentation. Fas is activated by binding to Fas ligand or crosslinking with antibodies. Fas and/or FAF1 can be expressed as a fusion protein. In a particular embodiment, the cell expresses CD4/fas and HA epitope tagged FAF1 fusion proteins. An L cell expressing both these fusion proteins is specifically provided in the invention.

Brief Summary Text (17):
The invention further provides a pharmaceutical composition useful in the treatment of a disease resulting from dysregulated apoptosis, comprising a FAF1 polypeptide and a pharmaceutically acceptable carrier. Instead of the FAF1 polypeptide, the pharmaceutical composition can comprise an expression vector capable of expressing the FAF1 polypeptide in an affected cell. The diseases contemplated for such treatment include cancer, autoimmune disease and viral infections. For these diseases, a constitutively active FAF1 polypeptide can be provided in the pharmaceutical composition. In a different set of diseases comprising myocardial infarction, stroke and reperfusion injury, arrest or blockage of apoptotic cell death is targeted. In the latter set of disease conditions, it may be therapeutically effective to express a Fas-interacting domain polypeptide in the affected cell to block the interaction between endogenous Fas and FAF1, thus blocking FAF1-potentiated apoptosis. Also provided are methods of alleviating a patient suffering from the aforementioned diseases by administering to the patient, a therapeutically effective amount of the above pharmaceutical compositions.

Brief Summary Text (18):
Finally, the invention provides several methods of isolating a FAF1-interacting protein. One method requires contacting a cell lysate suspected of containing a FAF1-interacting protein with a FAF1 polypeptide and isolating any protein bound to the FAF1 polypeptide, as a FAF1-interacting protein. A second method comprises labeling the cellular proteins, activating Fas expressed on the cell, immunoprecipitating FAF1 from the cell lysate and identifying a labeled protein that coimmunoprecipitates with FAF1. In a third method, a peptide library is exposed to a FAF1 protein to allow one or more peptides to bind to the FAF1 protein. Any bound peptide will be isolated as a FAF1-interacting protein.

Detailed Description Text (2):
Fas antigen, a member of TNF/NGF (Tumor Necrosis Factor/Nerve Growth Factor) receptor family, is a cell surface protein that mediates apoptosis. Fas induces apoptosis when activated by Fas ligand (FasL) binding or anti-Fas antibody crosslinking (Nagata, S., Seminars in Immunol., 6:3-8 (1994)). Fas plays an important role in the development and function of the immune system (Nagata, supra, Lowin et al., Nature, 370:650-652 (1994)). Malfunction of the Fas system causes lymphoproliferative disorders and accelerates autoimmune diseases, whereas exacerbation of Fas activity causes tissue destruction.

Detailed Description Text (3):
A point mutation in the cytoplasmic domain of Fas (a single base pair change from T to A at base number 786), replacing isoleucine with asparagine, abolishes the apoptotic signal transducing property of Fas (Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)). Mice homozygous for this mutant allele (lpr.sup.cg /lpr.sup.cg mice) develop lymphadenopathy and an autoimmune disease that resembles human systemic lupus erythematosus (Watanabe-Fukunaga, supra). They produce large quantities of IgG and IgM including autoantibodies such as anti-DNA, and rheumatoid factor (Cohen et al., Annu. Rev. Immunol., 9:243 (1991)) and develop nephritis or arthritis. Patients have been described with phenotypes similar to that of lpr mice (Sneller et al., J. Clin. Invest., 90:334 (1992)) and patients with altered Fas were recently reported (Rieux-Laucat et al.,

Abstracts of the 12th European Immunology Meeting, Barcelona (European Federation of Immunological Societies) (June 1994)).

Detailed Description Text (4):
The present invention identifies a novel Fas-associated factor 1, termed FAF1. FAF1, which was isolated using the two-hybrid screen in yeast (Durfee et al., Genes Dev., 7:555-569 (1993), specifically interacts with the cytoplasmic domain of wild type Fas but not the lpr.sup.cg mutated Fas protein. This interaction occurs not only in yeast but also in mammalian cells. When expressed in L cells, FAF1 potentiated Fas-induced apoptosis. A search of available DNA and protein sequence data banks did not reveal any significant homology between FAF1 and other known proteins. Therefore, FAF1 is a novel protein that binds to the wild type but not the inactive point mutant of Fas; it potentiates Fas-induced cell killing and is a signal transducing molecule in the regulation of apoptosis.

Detailed Description Text (9):
The yeast two-hybrid system (Durfee et al., Genes Dev., 7:555-569 (1993)) can be utilized to detect proteins capable of interacting with a known protein. Briefly, the method is as follows. Plasmids are constructed to encode two hybrid proteins which are coexpressed in Saccharomyces cerevisiae. One hybrid consists of the DNA-binding domain of the yeast transcriptional activator protein GAL4, fused to the known protein; the other hybrid consists of the GAL4 activation domain fused to protein sequences encoded by a library of DNA fragments. Interaction between the known protein and a protein encoded by one of the library plasmids leads to transcriptional activation of a reporter gene containing a binding site for GAL4. A suitable reporter gene is the Saccharomyces cerevisiae HIS3 gene and the E. coli lacZ gene (encoding .beta.-galactosidase (.beta.-gal)). Yeast cells are tested for growth in media lacking histidine and for expression of .beta.-gal activity which can be assayed by detecting blue colonies on a plate containing the substrate 5-bromo-4-chloro-3-indolyl .beta.-galactoside.

Detailed Description Text (10):
In the present invention, the hybrid construct encoding the known protein consists of the .lambda. repressor dimerization domain/Fas cytoplasmic domain chimera fused to the DNA-binding domain of GAL4. Using this two-hybrid screening system, cDNA clones encoding a protein that interacted with the Fas fusion molecule were isolated. A probe prepared from the longer cDNA clone was used to screen a murine thymus cDNA library from which two full length cDNA clones encoding FAF1 were isolated.

Detailed Description Text (12):
As used herein, a "Fas-associated factor 1" is a protein which has an affinity for Fas and binds or physically interacts with Fas.

Detailed Description Text (13):
A "FAF1 interacting molecule" or "FAF1 associating molecule" is a molecule which has an affinity for FAF1 and binds or physically interacts with FAF1. If the interacting molecule is a protein, it is referred to herein as "FAF1 interacting protein". The interaction can be transient, lasting only a fraction of a second or it can be stable so as to enable the detection of the complex of FAF1-FAF1 interacting molecule. Preferably, this interaction persists for at least ten seconds, ideally several minutes. The term "FAF1 interacting molecule" does not imply any particular molecular size or other structural or compositional feature other than that the molecule or compound in question is capable of binding or otherwise interacting with FAF1. The interacting molecule may be a substrate of FAF1, an enzyme that acts on FAF1, a protein that FAF1 is involved in localizing, an effector molecule of FAF1 and/or Fas or a molecule that alters the conformation of FAF1 upon interaction. Interacting or associating molecules that can be investigated by this invention include but are not restricted to agonists and antagonists of FAF1, cellular proteins encoded by oncogenes or proto-oncogenes, lipids, toxins, hormones, sugars, cofactors, peptides, proteins, enzyme substrates, drugs and compounds from plant or animal sources.

Detailed Description Text (14):
A "Fas-interacting domain polypeptide or peptide of FAF1" is defined as a polypeptide or peptide having a sequence corresponding to a region in the wild-type FAF1 protein, which physically interacts with the cytoplasmic region of Fas. The peptide will typically be in the range of 10-30, preferably 25, amino acids.

Detailed Description Text (24):
As used herein, "constitutively active FAF1" means that FAF1 is functionally active independent of prior activation of Fas. Cells expressing the constitutively active FAF1 will exhibit induced apoptosis without prior activation of Fas by Fas ligand binding or Fas cross-linking. "Constitutively active FAF1" shall be deemed to include functional derivatives thereof or homologs thereof of the wild-type FAF1 protein. Derivatives can be produced by modifying any region in the wild-type protein such as by deleting negative regulatory regions or by making amino acid substitutions.

Detailed Description Text (28):
The present invention provides an "isolated nucleic acid" encoding a novel Fas-associated factor 1, defined herein as FAF1. The nucleotide sequence of the cDNA encoding full length FAF1, SEQ ID NO:1, is shown in FIG. 2A and the amino acid sequence,

SEQ ID NO:2, is shown in FIG. 2B.

Detailed Description Text (32):
In a preferred embodiment, the FAF1 polypeptide or fragment thereof is capable of associating with the cytoplasmic domain of Fas. In addition, pharmaceutical compositions are provided that include the FAF1 polypeptide and its derivatives with a pharmaceutically acceptable carrier.

Detailed Description Text (38):
A gene that hybridizes with the probe and is determined to be substantially homologous to the FAF1 gene in nucleotide sequence will be isolated. The homologous gene will be inserted into an appropriate expression vector and introduced into a suitable host for expression to produce the encoded polypeptide. The encoded polypeptide will then be assayed to determine if it associates with the appropriate species of Fas and functions like mouse FAF1, using the same procedure for analyzing the interaction of FAF1 with Fas, described below in the Experimental Examples.

Detailed Description Text (39):
Other FAF1 structurally- or functionally-related family member proteins will associate and interact with receptors similar to Fas and regulate apoptosis in other systems. The same library screening approach can also be used to identify other members of the family that share "substantial homology" with FAF1.

Detailed Description Text (42):
Another aspect of the invention is the mapping of the functional domains of FAF1. Functional domains of FAF1 include regions of FAF1 that: interact with Fas; interact with effector molecules; exhibit an enzymatic activity; or regulate the activity of FAF1 itself. Manipulating these domains of FAF1 can provide a means by which to control Fas and FAF1 mediated apoptosis. For example, polypeptides or peptides corresponding to the Fas-interacting domain of FAF1 can be used to block Fas mediated apoptosis. Such polypeptides will generally be in the range of 6 to 100 amino acids. Circumstances whereby blocking of apoptotic cell death is desirable are described below under therapeutic uses.

Detailed Description Text (43):
There may be negative regulatory regions in FAF1 that control the activation of FAF1 so that premature or unnecessary cell death does not occur. These regulatory regions also serve as useful targets for mutagenesis to affect the Fas apoptotic signaling pathway. For example, a constitutively active FAF1 could be produced by removing a negative regulatory region. Such a modified FAF1 can be selectively expressed in targeted population of cells to affect cell death. This approach can be used to target elimination of cells that exhibit uncontrolled proliferation such as cancerous cells and autoreactive cells.

Detailed Description Text (45):
Generally, the mutants will have overlapping deletions. Initially, mutants will be created that have deletions typically in the range of 10-50 amino acids. For subsequent more precise mapping, the deletions in a particular region will be smaller, typically in the range of 5-10 amino acids. Mutants having single amino acid changes will be useful to define key residues that determine structure or that may be involved in physical contact with Fas or a downstream interacting or effector molecule of FAF1. Such mutants can be generated by known techniques such as by site-directed mutagenesis or by PCR.

Detailed Description Text (47):
The mutant transfected FAF1 will be analyzed for various characteristics that indicate increased, reduction or complete loss of Fas-mediated function. For example, mutant FAF1 will be analyzed for physical association with Fas by its ability to coimmunoprecipitate with Fas or a Fas fusion polypeptide. The extent to which mutant FAF1 can potentiate Fas-mediated apoptosis will be quantitated. Such assays are described in more detail under Experimental Examples below.

Detailed Description Text (55):
Fusion proteins encoded by FAF1 fused to the GAL4 DNA binding domain can be used in the yeast two hybrid system to isolate any proteins other than Fas that interact with the FAF1 protein, in particular, proteins that act downstream of FAF1. The yeast two-hybrid system is described above and in the experimental examples. This method allows the isolation of the cloned genes for the interacting proteins and eventually the identification of the interacting proteins. Knowledge of the interacting proteins in the Fas signaling pathway will allow the screening of drugs for agonists and antagonists of Fas dependent apoptosis.

Detailed Description Text (56):
The nucleic acid constructs will be useful to introduce into cells, providing an efficient and economical means to produce commercially useful quantities of the protein compositions. Transfected cells producing varying quantities of full length FAF1 will also be useful in evaluating the effect of overexpression of FAF1 on Fas function and apoptosis. Nucleic acid constructs expressing various lengths and mutant forms of FAF1 can be used to determine structure-function relationships.

Detailed Description Text (62):

Another aspect of the invention relates to methods of screening for agonists and antagonists of FAF1 which can affect apoptosis mediated by the Fas pathway. The agonists and antagonists are preferably small molecules defined as being in the range of MW 5-20 kD and can cross the plasma membrane or be taken up by the cell. Test molecules will include oligonucleotides, lipids, toxins, hormones, sugars, cofactors, peptides, small proteins, drugs and compounds from plant or animal sources and recombinantly produced substances. FAF1 transfected cells (FAF1 transfectants) are useful for such screening although cells naturally expressing Fas and FAF1 can also be used.

Detailed Description Text (63):
The screening method involves contacting a cell expressing both Fas and FAF1 with a test molecule, activating Fas, and analyzing the cell for any effects on apoptosis, increased apoptosis indicative that the test molecule is an agonist and decreased or loss of apoptosis indicative that the test molecule is an antagonist. Fas can be activated by providing Fas ligand to the cell culture media or solution of the cell sample, to bind the Fas receptor on the cell surface. Alternatively, Fas can be activated by crosslinking with antibodies to the extracellular domain of Fas. If Fas is expressed as a fusion protein, an antibody specific to the extracellular domain portion of the fusion protein will be used to crosslink Fas. For example, an anti-CD4 antibody can be used to crosslink a CD4/Fas fusion protein. FAF1 can be naturally or recombinantly produced in the wild type form or expressed as a fusion protein. Appropriate positive and negative control cells will be required for comparison.

Detailed Description Text (64):
In one embodiment, L cells expressing a Fas fusion protein, such as the CD4/fas-16 cells described in the Experimental Examples, are further transfected with FAF1 or mock transfected with vector as described. The mock transfected CD4/fas-16 serve as negative control for the functional assays. Transfectants expressing both Fas and FAF1 (test cells) and mock transfectants expressing Fas only (negative control cells), are contacted with a solution sample containing one or more test compounds. A sample of double transfectants will be treated under the same conditions but in the absence of the test molecule. The cells can be exposed to the test compound added to the culture media. The test compounds can initially be screened in pools comprising about 2-10 different compounds or molecule types per sample solution.

Detailed Description Text (65):
Cells treated in the absence or presence of the test Molecule will be tested for e.g. for disruption of Fas-FAF1 interaction or for any effect on apoptosis with or without stimulation of Fas. In one embodiment, the cells are crosslinked with anti-CD4 antibody (e.g., L3T4) and then assayed for any effects on FAF1 activity essentially as described in the Experimental Examples under the subsection Association of FAF1 with Fas. The cells are analyzed for morphological changes such as cell membrane blebbing and other characteristics of apoptosis. Cellular DNA can be analyzed for fragmentation by gel electrophoresis. Based on these criteria, the percentage of apoptotic cells in the test cells as compared to similar cells treated in the absence of the test molecule or the mock transfected cells can be determined. The test molecule is considered an agonist if the test cells contacted with the test molecule show increased frequency of apoptosis over the negative control. In contrast, loss of or reduction in apoptosis in the test cells is indicative that the test molecule is an antagonist.

Detailed Description Text (71):
The polypeptide compositions of FAF1 are useful for raising antibodies, both polyclonal and monoclonal. Such antibodies are powerful tools that can be employed in various assays and diagnostic situations particularly where immunoprecipitation, immunoblotting and affinity purification procedures are necessary. Thus, one aspect of the invention is to provide antibodies that specifically bind to the FAF1polypeptide comprising the sequence of SEQ ID NO:2, an allelic or species variation thereof, or a fragment thereof. Antibodies capable of specifically blocking the binding of FAF1 to Fas are also desirable reagents. The invention provides a rabbit antiserum produced using the entire FAF1 as immunogen. This rabbit anti-FAF1 antiserum is useful in both immunoprecipitating FAF1 and detecting FAF1 on Western blots. The invention also provides hybridoma lines that produce monoclonal antibodies to the FAF1 polypeptide.

Detailed Description Text (73):
FAF1 antibodies are also useful to study the interaction of FAF1 with Fas in vivo, in normal and growth dysregulated or cancerous cells. The same protocol described in the experimental examples for studying the interaction of FAF1 with Fas in intact cells can be followed.

Detailed Description Text (74):
Thirdly, the FAF1 specific antibodies find use in isolating any FAF1 associating protein that co-immunoprecipitates with FAF1. FAF1 associating proteins are useful to study the downstream effectors of Fas and the regulation of Fas and FAF1 function.

Detailed Description Text (77):
For immunization, preferably peptides corresponding to regions of the protein comprising hydrophilic residues or residues exposed to the aqueous phase are selected. Immunogens comprising peptides corresponding to the region in FAF1 that interacts with Fas are desirable. Synthetic peptide fragments may be prepared in a peptide synthesizer and coupled to a carrier molecule (e.g., keyhole limpet hemocyanin) and the conjugate injected into rabbits at selected times over several months.

Detailed Description Text (91):
In a second method, FAF1 expressing cells, preferably transfectants that overexpress FAF1, are biosynthetically labeled in order to label the proteins. For this method, the cells to be transfected with FAF1 or FAF1 fragments are preferably cells that naturally express FAF1 so as to favor the likelihood of the cells also expressing a FAF1 associating protein. It is likely that the Fas pathway has to first be activated to induce a FAF1 interacting protein to associate with FAF1. Therefore, a sample of cells treated to activate Fas will be tested in parallel. As mentioned earlier, Fas can be activated by binding to Fas ligand (FasL) or by anti-Fas antibody cross-linking. If CD4/fas fusion protein expressing transfectants (such as CD4/fas-16 described below) are used, Fas can be cross-linked using anti-CD4 antibodies (e.g., L3T4). The cells are lysed under different detergent conditions and FAF1 is immunoprecipitated from the labeled cell lysate using anti-FAF1 antibody or antiserum. Labeled proteins that co-immunoprecipitate with FAF1 are identified by SDS-PAGE followed by autoradiography.

Detailed Description Text (98):
The invention also provides a method to block or modulate FAF1's ability to potentiate Fas-mediated apoptosis in vitro and in vivo. The method involves using peptides corresponding to the Fas-interacting domain of FAF1 to compete with endogenous wild-type FAF1 for associating with Fas. The Fas-interacting domain or region of FAF1 is determined as described above. By competing for binding to Fas, the Fas-interacting domain peptide will block or reduce the ability of wild-type FAF1 to interact with downstream effector molecules. This, in turn, will affect the cellular signalling events downstream of Fas.

Detailed Description Text (99):
The method of blocking Fas activity comprises providing a Fas-interacting domain peptide of FAF1 in a cell expressing Fas and FAF1 wherein the Fas-interacting peptide will bind to the Fas protein to block Fas activity. The Fas-interacting domain peptide itself can be directly introduced into the cell under study where arrest or modulation of Fas function is desired. Methods of introducing the Fas-interacting domain peptide into the cell include microinjection of the isolated peptide (expressed in other cells) or the use of appropriate drug delivery vehicles such as liposomes to deliver the polypeptide.

Detailed Description Text (100):
Alternatively, the Fas-interacting domain peptide can be provided by introducing an expression construct encoding the Fas-interacting domain peptide into the desired cell wherein the Fas-interacting domain peptide will be expressed in an amount effective to interfere with Fas activity. Expression constructs can be targeted to a particular cells by using nucleic acid delivery vehicles that contain targeting moieties on the surface of the vehicle. Examples of such vehicles include liposomes or recombinant viruses expressing receptors for cell surface markers. In some circumstances, complete blockage of Fas activity may require high level expression i.e. overexpression of the Fas-interacting domain peptide for effective competition. In that circumstance, the expression construct can be designed to contain the necessary elements such as strong promoters, inducible promoters and enhancers to achieve high level expression of the Fas-interacting domain peptide. The Fas-interacting domain peptide expressed intracellularly will be contacted with and bind to the Fas protein.

Detailed Description Text (104):
Several approaches for inducing or enhancing apoptosis are contemplated in the present invention. One approach is to bypass Fas by providing a constitutively active FAF1 that is functional in activating downstream events leading to apoptosis, independent of Fas activation by Fas ligand binding or Fas antibody cross-linking. The generation of a constitutively active FAF1 has been described above. The lpr.sup.cg /lpr.sup.cg mouse and human patients that manifest the lpr phenotype can be used here as a model. Due to a mutation in Fas, the lprcg/lprcg mice develop lymphadenopathy and autoimmune disease and produce large quantities of autoantibodies. The autoreactive B cells or certain subsets of these B cells can be targeted for apoptotic cell death by introducing a constitutively active FAF1 into these cells.

Detailed Description Text (108):
The next set of diseases are associated with accelerated rates of physiological cell death and characterized by cell loss. In these diseases, it would be desirable to provide therapeutic intervention to block or modulate the exacerbated Fas-mediated apoptosis. The disease conditions include ischemic injury such as caused by myocardial infarction (smooth muscle and epithelial cell death), stroke induced neuron death and reperfusion injury; AIDS; and liver disease caused by viral infection, such as fulminant hepatitis.

Detailed Description Text (109):
Methods to block the activation of FAF1 through Fas include the use of (i) antagonists that are small molecules, (ii) peptide inhibitors and (iii) antisense inhibition. Again, liposomes can be used to target delivery of the small molecular and peptide inhibitors as well as antisense nucleic acids to specific cell populations.

Detailed Description Text (110):
Small molecule antagonists and peptide inhibitors have been discussed above. Peptide inhibitors include peptides corresponding to regions of FAF1 that interact with Fas or with effector molecules of FAF1 or that exhibit an enzymatic activity. Peptide

inhibitors are intended to inhibit interactions between Fas and FAF1, Fas and effector molecule or Fas and substrate.

Detailed Description Text (112):
The disease conditions described such as exacerbated cell death due to myocardial infarction or stroke can be treated by administering to the patient, a therapeutic formulation comprising an inhibitor of FAF1 activity in an amount effective to block the FAF1 activity in affected cells in the patient. The inhibitor of FAF1 activity can be a Fas-interacting domain peptide. The patient can be administered a therapeutically effective amount of a pharmaceutical composition comprising a Fas-interacting domain peptide, and a pharmaceutically acceptable carrier. The Fas-interacting domain peptide will be specifically targeted to the affected cells, such as cardiac smooth muscle cells and epithelial cells in myocardial infarction, hepatocytes in fulminant hepatitis, and T cells and macrophages in AIDS. Another pharmaceutical composition for use in the treatment method will comprise an expression vector suitable for introduction into and expression of a therapeutically effective amount of a Fas-interacting domain peptide in these aforementioned cells.

Detailed Description Text (125):
DNA Constructs--A chimeric molecule of CD4 and fas was subcloned into vector PSM described in Brodsky et al., J. Immunol., 144:3078-3086 (1990), which has an SV40 replication origin and an SV40 early promoter. PSMCD4/fas contains the chimera with a wild type cytoplasmic domain of Fas. PSMCD4/fas786A has a T to A point mutation at base pair 786 in the cytoplasmic domain of Fas. Fusion molecules of the .lambda. repressor dimerization domain and Fas cytoplasmic domain were inserted in frame with GAL4 DNA-binding domain and HA epitope in the vector PAS-CHY (Durfee et al., Genes Dev., 7:555-569 (1993)). FAF1 tagged with an HA epitope at the N-terminus was subcloned into the PCGN vector (with a CMV promoter) to make PCGN8.1.

Detailed Description Text (132):
Fas-Mediated Apoptosis

Detailed Description Text (133):
In order to understand the mechanism of signal transduction in Fas-mediated apoptosis, it was first determined whether the cytoplasmic domain is sufficient to initiate an apoptosis signal. A chimeric cDNA (CD4/fas) containing the cytoplasmic domain of murine Fas linked to the extracellular and transmembrane domains of murine CD4 (CD4/fas) was made. As control, the point mutation of Fas in lpr.sup.cg mice (T786A) was also made as an analogous chimeric molecule (CD4/fas786A). The chimeras were stably transfected into L cells and clones expressing equivalent levels of wild type (CD4/fas) or mutant (CD4/fas786A) chimeric molecules were chosen for analysis (FIG. 1A). L cells expressing CD4/fas (CD4/fas-16 underwent apoptotic cell death when crosslinked by monoclonal antibody against CD4 (L3T4, Caltag)) in the presence of actinomycin D (Itoh et al., DNA Cloning, a Practical Approach, IRL Press, Oxford. Vol. II, pp. 143-190 (1991). DNA fragmentation, characteristic of apoptosis, was observed two hours after the antibody crosslinking (FIG. 1B). Cells were shrunk and detached from the bottom of the culture dish at 10 hours (FIG. 1C). However, L cells expressing the mutant chimera (CD4/fas786A-23), under the same treatment, did not undergo apoptotic cell death. Multiple clones of each type were analyzed and gave the same results. It was concluded that the cytoplasmic domain of Fas can initiate an apoptotic signal.

Detailed Description Text (134):
The results also showed that dimerization of the Fas cytoplasmic domain is sufficient to generate an apoptotic signal. The antibody L3T4 (GK1.5), used here to induce apoptosis through the Fas cytoplasmic domain, is a bivalent Rat IgG-2b. As shown in FIG. 1B, there was no significant change upon the addition of a secondary anti-Rat IgG antibody, again indicating that dimerization was sufficient for Fas activation.

Detailed Description Text (135):
Screening for Fas-Interacting Protein

Detailed Description Text (136):
An improved version of the yeast two-hybrid system (Durfee et al., Genes Dev., 7:555-569 (1993) originally devised by Fields and Song, Nature, 340:245-246 (1989)) was used to screen for Fas interacting proteins. In order to simulate activated dimeric Fas, a fusion molecule of the .lambda. repressor dimerization domain and the Fas cytoplasmic domain was constructed. The fusion molecule was then linked to the DNA binding domain of GAL4 for two-hybrid screening. As a control, a similar construct was made with the T786A mutation (lpr.sup.cg mutation) in the Fas cytoplasmic domain.

Detailed Description Text (137):
The nucleotide and amino acid sequence of murine and human Fas is provided in Watanabe-Fukunaga et al., J. Immunol., 148:1274-1279 (1992) and Itoh et al., Cell, 66:233-243 (1991), respectively. The Fas cDNA sequence of lpr.sup.cg mice is disclosed in Watanabe-Fukunaga et al., Nature, 356:314-317 (1992). The amino acid sequence of the Fas cytoplasmic domain from wild-type mouse, cg mouse and human can also be found in Watanabe-Fukunaga et al., Nature, supra. .lambda. repressor dimerization domain sequence is disclosed in Amaya et al., Development, 118:477-487 (1993).

Detailed Description Text (139):
The plasmid pAS1.FasCyt contains the GAL4 DNA binding domain fused to the Fas cytoplasmic domain. The BamHI-EcoRI blunt-ended fragment containing the .lambda. repressor dimerization domain was inserted into pAS1.FasCyt at a blunt-ended NdeI site in between the GAL4 domain and the Fas cytoplasmic domain, producing an in-frame fusion of GAL4 DNA binding domain-.lambda. repressor dimerization domain-Fas cytoplasmic domain in the construct pAS1/RDD/CYT. This ligation creates 4 new amino acids, RSPL (SEQ ID NO:3), at the junction of GAL4 and .lambda. dimerization domain, and 6 new amino acids, GCRNSI (SEQ ID NO:4), between the dimerization domain and the Fas cytoplasmic domain. As a control, a similar construction was made using Fas having the T786A mutation (lpr.sup.cg mutation) in the cytoplasmic domain.

Detailed Description Text (140):
A GAL4 transactivation domain-tagged cDNA expression library was prepared using a murine T cell line cDNA library (a gift from Dr. S. Elledge). The yeast strains and vectors used herein as well as cDNA libraries from different cell lines, linked to the GAL-4 transactivation domain, are also commercially available from, e.g., Clontech Labs Inc., Palo Alto, Calif. The yeast reporter strain is cotransformed with the .lambda./Fas/GAL4 DNA binding domain fusion construct and the T cell cDNA library/GAL4 transactivation domain fusions.

Detailed Description Text (141):
More than 1.1 million clones of the T cell line cDNA library were screened for their ability to interact with the Fas fusion molecule in the two-hybrid system. Four independent clones interacted specifically with the wild type Fas constructs but not the mutant Fas constructs. Two clones had 2.2 kb inserts and two other had 2 kb inserts. Sequence analysis showed that all 4 clones were derived from the same gene and fused to the activation domain of GAL4 in the same reading frame. The inserts of the shorter 2 kb clones were missing approximately 150 bp at the 5' end and 50 bp at the 3' end of the sequence of the longer clones. A murine thymus cDNA library (a gift of Dr. M. Davis) was screened with a DNA probe consisting of 0.7 kb of the most 5' end of the 2.2 kb clone isolated by the two-hybrid screening in yeast. Two independent cDNA clones of approximately 2.6 kb were obtained from the murine thymus cDNA library.

Detailed Description Text (143):
Sequence analysis indicated that these were full length cDNAs and contained an open reading frame encoding a protein of 649 amino acids (FIG. 2A). The deduced molecular weight is 74 kD and the PI was 4.6. The translation start site contains a perfect "Kozak" consensus sequence (Kozak, M., J. Cell Biol., 108:229-241 (1989)). There are two regions, amino acids 280 to 310 and 490 to 590, that are highly negatively charged and have a predicated .alpha.-helical secondary structure. There are three potential myristoylation sites located at amino acid 50, 306 and 310. There are also three N-glycosylation sites at amino acid 163, 209 and 423. No significant sequence homology of these clones was found with any complete protein sequence in available sequence data banks. This novel protein was named Fas-associated factor 1 (FAF1).

Detailed Description Text (144):
Association of FAF1 with Fas in Mammalian Cells

Detailed Description Text (146):
FAF1 was expressed equally in the Cos cells expressing either CD4/fas or CD4/fas786A (FIG. 3A). By immunoprecipitating with anti-CD4 antibody and blotting for the HA epitope, a much greater quantity of FAF1 was co-immunoprecipitated with CD4/fas than with CD4/fas786A (FIG. 3B), although more CD4/fas786A was immunoprecipitated than CD4/fas (FIG. 3C). As shown in FIG. 3B, the molecular weight of FAF1 detected on SDS-PAGE is 75-80 kD which is heavier than the predicated 74 kD. This could be accounted for by the post-translational modifications such as glycosylations of the molecule. Thus, FAF1 was able to specifically associate with the cytoplasmic domain of wild type Fas in Cos cells.

Detailed Description Text (147):
To determine the significance of the association between FAF1 and Fas, FAF1 was transiently expressed in CD4/fas-16- or CD4/fas786A-23-stably transfected L-cells. CD4/fas-16 and CD4/fas786A-23 cells were co-transfected with PSV-.beta. Gal and PCGN8.1 or PCGN alone (1:5 ratio) by DEAE Dextran method (Gorman, DNA Cloning, a Practical Approach, IRL Press, Oxford. Vol. II, pp. 143-190 (1985)) or Lipofectomin (BRL). Forty-eight to seventy-two hours later, transfectants were crosslinked by different amounts of L3T4 (GK1.5) antibody in the presence of actinomycin D: 1 .mu.g/ml (FIG. 4 C, F, I, and L) or 200 ng/ml (B, E, H and K) of L3T4 or a control rat IgG (A, D, G and J) as described in FIG. 1B. The cells were fixed and photographed one hour later.

Detailed Description Text (148):
In CD4/fas-16 cells, transient expression of FAF1 resulted in more rapid and extensive apoptosis than in mock transfected cells (FIG. 4A). One hour after addition of 200 ng/ml of L3T4 crosslinking antibody, approximately 60% of CD4/Fas-16 cells expressing FAF1 had undergone apoptotic cell death compared with 30% in the cells without FAF1 overexpression (FIG. 5). Increasing the L3T4 concentration to 1 .mu.g/ml, increased the apoptotic cell death to approximately 70% and 40% respectively

(FIG. 5). There was no obvious apoptosis observed in CD4/fas786A-23 cells treated similarly (FIGS. 4 and 5). Apoptosis induced through Fas was thus increased from 30%-40% in the controls to 60%-70% when FAF1 was expressed. Similar results were obtained in a human T cell leukemia line, Jurkat, where transient expression of FAF1 potentiated apoptosis induced by anti-human Fas antibody. These data indicate that FAF1 can potentiate apoptosis mediated by Fas and acts downstream of Fas.

Detailed Description Text (149):
The results indicate that FAF1 is a molecule which acts downstream in the Fas signal transduction pathway. FAF1 can interact selectively with the wild type cytoplasmic domain of Fas. This specific interaction occurred not only in yeast cells but also in mammalian cells. The binding is not the result of overexpression of FAF1 in Cos cells because the level of FAF1 expression was relatively low (approximately one tenth) compared to other proteins that were expressed with the same antibody tag. When expressed transiently in L cells, FAF1 was able to potentiate apoptosis induced by Fas.

Other Reference Publication (7):
Itoh et al., "The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis," Cell, 66:233-243 (1991).

Other Reference Publication (9):
Nagata, "Mutations in the Fas antigen gene in lpr mice," Immunol., 6:3-8 (1994).

Other Reference Publication (10):
Sato et al., "FAP-1: a protein tyrosine phosphates the associates with Fas," Science, 268:411-415 (1995).

Other Reference Publication (11):
Watanabe-Fukunaga et al., "The cDNA structure expression, and chromosomal assignment of the mouse Fas antigen," J. Immunol., 148(4):1274-1279 (1992).

Other Reference Publication (12):
Watanabe-Fukunaga et al., "Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis," Nature, 356:314-317 (1992).

Other Reference Publication (13):
Boldin, et al., "A Novel Protein that Interacts with the Death Domain of Fas/APO1 Contains a Sequence Motif Related to the Death Domain", J. Biol. Chem, 270(14):7795-7798 (1995).

Other Reference Publication (14):
Chinnaiyan, et al., "FADD, a Novel Death Domain-Containing Protein, Interacts with the Death Domain of Fas and Initiates Apoptosis", Cell, 81:505-512 (1995).

Other Reference Publication (15):
Chu, et al., "A Novel Protein, FAP, Potentiates Fas-mediated Apoptosis", Journal of Investigative Medicine, 43(suppl. 2):289A (1995).

Other Reference Publication (19):
Stanger, et al., "RIP: A Novel Protein Containing a Death Domain That Interacts with Fas/APO-1 (CD95) in Yeast and Causes Cell Death", Cell, 81:513-523 (1995).

CLAIMS:

1. An isolated polypeptide comprising a domain of a Fas-associated factor 1 (FAF1) polypeptide, said polypeptide capable of associating with a cytoplasmic domain of Fas, wherein said FAF1 domain is encoded by a nucleic acid sequence that comprises at least 18 nucleotides and hybridizes under stringent conditions to the complementary nucleic acid sequence shown in SEQ ID NO: 1 or to a degenerate form thereof.

4. A kit useful for the prophylactic or therapeutic treatment of a disease resulting from dysregulated Fas-associated factor 1 (FAF1) polypeptide-potentiated apoptosis comprising a polypeptide of claim 1.

5. A pharmaceutical composition useful in the treatment of a disease resulting from dysregulated Fas-associated factor 1 (FAF1) polypeptide-potentiated apoptosis, comprising an isolated polypeptide comprising a domain of an FAF1 polypeptide, said polypeptide capable of associating with a cytoplasmic domain of Fas, wherein said FAF1 domain is encoded by a nucleic acid sequence that comprises at least 18 nucleotides and hybridizes under stringent conditions to the complementary nucleic acid sequence shown in SEQ ID NO:1 or to a degenerate form thereof, and a pharmaceutically acceptable carrier.

7. The fusion protein of claim 6, wherein the fusion protein is FAF1-GAL4 activation domain.